


Anja Bosserhoff
Editor

Melanoma Development

Molecular Biology, Genetics
and Clinical Application

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and Clinical Application**

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Preface

This book, *Melanoma Development: Molecular Biology, Genetics and Clinical Application*, provides a comprehensive insight into the molecular changes of malignant melanoma and implications for therapeutic approaches. It summarizes the current knowledge on the nature of melanoma and is intended to provide a broad overview of major aspects of the molecular biology of melanoma.

The chapters that comprise this book review what is currently known about the molecular basis of melanoma development and progression. The introduction gives a brief overview of the clinical nature of melanoma, and then general mechanisms of melanoma growth and development, as well as new research findings, are discussed. It addresses different molecular aspects of melanocyte and melanoma biology, from embryology to therapies. Through this comprehensive approach, it provides a cohesive view of melanoma biology that may serve as a source of information and starting point for future investigations, hopefully leading to more rapid clinical breakthroughs. By summarizing the newest data and presenting upcoming areas of research in the field, the book will be a valuable reference for biologists, basic scientists, and physicians working in the important fields of melanoma, cancer research, therapy, dermatology, and surgery. Our current knowledge and future understanding of the molecular mechanisms involved in cell transformation and tumor progression will soon lead to sophisticated targeted therapies.

I am grateful to all the authors for their highly interesting, forward-looking contributions, and for their support in this book project. I am optimistic that this book will add to the continuing education of scientists in the field of melanoma research. Recent studies with targeted B-RAF inhibitors have given us the grounds to hope that these therapies will be successful. *Melanoma Development: Molecular Biology, Genetics and Clinical Application* aims to contribute to this body of knowledge.

Regensburg, Germany

Anja Bosserhoff

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1.1

Clinicopathologic Overview of Melanoma

Human melanoma is the most rapidly increasing malignant skin disease in Caucasians (Leiter and Garbe 2008). Once considered a rare disease, the lifetime risk for developing melanoma in the US has increased from approximately 1 in 1,500 during the 1930s to its present risk of approximately 1 in 60 (Giblin and Thomas 2007). The American Cancer Society's recent cancer report estimates that 68,130 new cases of melanoma will be diagnosed and 8,700 deaths will result from melanoma during 2010 in the United States (American Cancer Society 2010). Important risk factors for developing melanoma include increased number of melanocytic nevi, a family history of melanoma, or a history of previous melanoma (Seykora and Elder 1996; Psaty et al. 2010). Prolonged sun exposure associated with increased outdoor activity has been suggested to play an important role in the epidemiologic increase in the incidence of melanoma (Leiter and Garbe 2008; Moan et al. 2008). Acute exposure of the skin to ultraviolet (UV) radiation can induce varying degrees of erythema, pigmentation, and impairment of immune function (Matsumura and Ananthaswamy 2004). Recently, increased numbers of melanocytic nevi associated with sunburn and intermittent or "holiday" sun exposure has been suggested as a major risk factor for developing melanoma in different studies (Elwood and Jopson 1997; Newton-Bishop et al. 2010). In fact, the Clark model for melanoma suggests a stepwise progression from hyperplastic and dysplastic nevi to melanoma (Fig. 1.1) (Clark et al. 1984).

From a clinical perspective, melanocytic nevi are benign proliferations that appear as flat or slightly raised pigmented growths generally found on sun-exposed skin. Histologically, these are formed by proliferating melanocytes that gradually assume a more round or oval shape from their normal dendritic-like morphology, forming nests along the basal layer and growing toward the dermis as cords. As the nevus cells grow into the dermis, melanin production significantly decreases as tyrosinase activity is progressively

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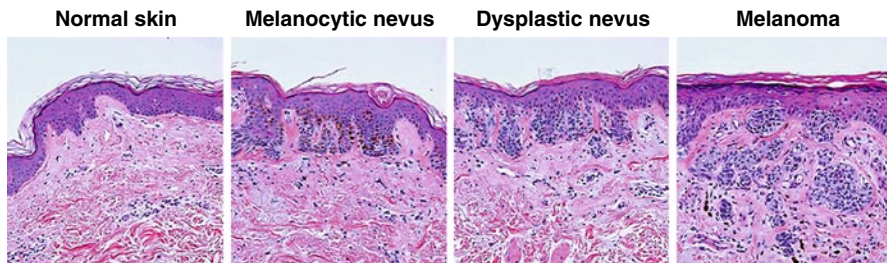


Fig. 1.1 Histological model for melanoma progression. It is believed that melanoma originates from benign pigmented lesions called melanocytic nevi formed by proliferating melanocytes generally associated with exposure of normal skin to ultraviolet radiation from the sun. These nevi can begin to show cytoplasmic and nuclear alterations typical of dysplasia. As the degree of dysplasia increases so does the chances for malignant transformation of these lesions into melanoma (Original magnification $\times 20$)

lost in a process known as “maturation”. Dysplastic nevi can progress from preexisting benign nevi or form *ex novo* in a new location. Clinically, they are larger than most benign nevi, have irregular borders, and appear with varying degree of pigmentation. Dysplastic nevi are composed of irregularly shaped cells with hyperchromic nuclei. Discordance in the diagnosis of benign, dysplastic, and melanoma based on morphology alone, however, has continued to plague even experienced pathologists (Ackerman 1996; Lodha et al. 2008; Shoo et al. 2010). Different molecular biomarkers have been proposed to help to differentiate benign nevi from malignant melanomas and are still being validated. Recent studies, for example, have claimed high success rates in discriminating benign lesions from melanoma with the use of multiple tissue marker arrays (Kashani-Sabet et al. 2009) or different fluorescence in situ hybridization (FISH) DNA probes (Gerami et al. 2009). The ideal goal would be to develop high throughput analytical systems that would increase the feasibility of adopting such multimarker approaches at all diagnostic centers.

There remains the fact that some melanomas can also form in areas of the body not exposed to the sun, such as mucous membranes (DeMatos et al. 1998; Das et al. 2010) or arise independently of previous nevi suggesting that other factors, alone or in combination, are also involved in the pathogenesis of melanoma. Recent advances in the understanding of the different cellular signaling events in melanoma have shed some light on the identification of potential underlying molecular mechanisms. To this regard, downstream signaling events caused by mutations in NRAS and BRAF as well as PI3K/Akt, MAPK/ERK, and c-KIT activity have been found to play a role in melanoma signaling (Kyrgidis et al. 2010). Since exposure to UV radiation can lead to DNA damage, sun exposure may represent an obvious cause of these mutations. However, the identification of some of these genetic mutations in congenital nevi as well seems to argue against the fact that UV radiation is the sole culprit of these mutations. As mentioned previously, a family history of melanoma is an important predictor of melanoma risk (Psaty et al. 2010), indicating that genetic predisposing factor(s) must also play a role during melanomagenesis, as for instance, with CDKN2A, where up to 40% of members of melanoma-prone families show germ-line mutations in this tumor suppressor gene (Hansson 2010).

As described in the Clark model for melanoma progression, early melanoma is characterized by localized growth referred to as “radial growth phase” or “thin melanoma”. During this phase, the melanoma cells tend to grow between the layers of the epidermis and superficial dermis with significantly low risk for metastasis. Surgical excision of these relatively flat lesions is associated with high cure rates. With time, the melanoma assumes a more nodular appearance as the relatively larger and irregular melanoma cells begin to penetrate vertically and invade the dermis (“vertical growth phase”). The depth of invasion of the melanoma related to the anatomical structures of the dermis and subcutaneous tissue or measured directly in millimeters is used to predict clinical outcome with deeper (or thicker) lesions associated with reductions in overall patient survival (Balch et al. 2009). Other factors, such as the presence of tumor ulceration or increased mitotic rates, also negatively affect prognosis (Balch et al. 2009). The metastatic process of melanoma is facilitated once melanoma cells begin to invade vascular and lymphatic structures. At this point chemotherapy is the therapeutic option of choice. Current treatment strategies for advanced-stage melanoma employing cytotoxic agents are often accompanied by important side effects and associated with relatively low percentages of objective response rates (Atallah and Flaherty 2005). Similarly, molecular redundancy and cross talk between multiple signaling pathways appear to have undermined the efficacy hoped to achieve with targeted molecular biotherapy. Generally, anticancer drugs appear to have some initial effect usually due to killing of the majority of the cancer cells sensitive to the chemotherapeutic agent. However, increased signaling of cell survival pathways, enhanced DNA repair mechanisms, or mutations of molecular targets in melanoma cells often lead to resistance to therapy. Attempts continue at identifying novel diagnostic markers and molecular targets important for melanomagenesis and disease progression. Work is also needed to detect and quantify chemoresistance and to better understand the molecular mechanisms that are involved in inducing drug resistance in melanoma. Results from these efforts could help to identify those patients most likely to present resistance to treatment and that would otherwise benefit from a combinatorial approach.

1.2

Chapters of the Book

In the individual chapters of this book all aspects of basic biology of melanoma are addressed. Further, general mechanisms and therapeutic approaches based on this knowledge are described.

The first chapter by Zalpha Abdel-Malek concentrates on melanocytes, the cellular origin of malignant melanoma. For a general understanding of the molecular processes in melanoma development, epidemiology, as illustrated in Chap. 3 by Marianne Berwick, is of major importance enhancing our knowledge of the tumor-inducing stimuli. Basic genetic and genomic changes are summarized in Chap. 4 by Göran Jönsson and Hensin Tsao.

The following chapters are focusing on changes in basic molecular regulation. Here, chapters on transcriptional regulation by Satoru Yokoyama and David Fisher, on miRNAs by Daniel Müller and Anja Bosserhoff, on Cell signaling by Eric Lau and Ze’ev A. Ronai,

on proteases by Paola Zigrino and Cornelia Mauch, and on molecules in cell–cell and cell–matrix contacts by Silke Kuphal and Nicolas Haass give insight into molecular details.

The next chapters summarize characteristics of molecular processes in melanoma. The chapter by Jürgen Eberle describes changes in apoptotic processes in malignant melanoma, and Helen Rizos and Lyndee L Scurr summarize the knowledge on the role of senescence.

As melanoma is a complex disease understanding is enforced by looking at general biological mechanisms and the analysis of other cell types than melanoma cells existing in a melanoma tumor. Chapters on melanoma stem cell by Markus Frank, on lessons from embryology by Luigi Strizzi, Mary Hendrix and others, and on the influence of the tumor microenvironment by Gao Zhang and Meenhard Herlyn are summarizing the available information.

A chapter by Birgit Schitteck and Thomas Tüting concentrates on model systems which are available in melanoma research.

The last two chapters comment on the current status of melanoma therapy: Keith Flaherty summarizes the new approaches targeting cellular signaling whereas the chapter of Alexander M.M. Eggermont, Caroline Robert, and Dirk Schadendorf focuses on the new era of immunotherapy in melanoma.

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Epidermal Melanocytes: Regulation of Their Survival, Proliferation, and Function in Human Skin

2

Zalfa A. Abdel-Malek and Viki B. Swope

Abstract Melanocytes are cells specialized in the synthesis of the pigment melanin, in the form of eumelanin, the brown/black, and pheomelanin, the red/yellow pigment (Ito and Wakamatsu 2003). Melanocytes reside in the cutaneous epidermis, within hair follicles, in the eye, the leptomeninges, the inner ear, and as has been demonstrated lately, in the heart (Brito and Kos 2008; Goldgeier et al. 1984; Tachibana 1999; Yajima and Larue 2008). Melanin produced by melanocytes provides the skin, hair, and eyes with their distinctive coloration. In this chapter we focus on epidermal melanocytes, since they have been the most investigated due to their importance in photoprotection against sun-induced skin cancers, and for being the precursors for cutaneous melanoma, the deadliest form of skin cancer. We hereby provide a brief summary of the properties of melanocytes, review how cutaneous pigmentation is regulated, and discuss the significance of paracrine and autocrine factors and their signaling pathways in modulating the survival, proliferation, and function of melanocytes, constitutively, and in response to solar ultraviolet radiation (UV), a major environmental stressor and etiological factor for skin cancers, including melanoma. We end by briefly describing how the knowledge gained about the regulation of melanocytes can be translated into preventative and therapeutic strategies for melanoma.

2.1 Unique Properties of Melanocytes

In the human epidermis, melanocytes reside on the basement membrane, at the epidermal-dermal junction (Jimbow and Fitzpatrick 1975). Melanocytes differ from keratinocytes, the main structural cells of the epidermis, in many respects. Unlike basal keratinocytes that are highly proliferative and capable of regenerating the epidermal layers, melanocytes

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have a low proliferation potential. Keratinocytes undergo a well-defined differentiation program that culminates in their death by an apoptosis-like process (reviewed by Eckert et al. 1997). On the other hand, most melanoblasts, the precursors for melanocytes, become fully differentiated upon reaching their final destination, the epidermis, after their migration during embryonic development from the neural crest (Bronner-Fraser 1993) (see also Chap. 13). Melanocytes are resistant to apoptosis, as they are endowed with antiapoptotic mechanisms, exemplified by constitutive expression of the antiapoptotic protein Bcl2, which enable them to survive (Plettenberg et al. 1995). Contrary to keratinocytes, melanocytes have a very long life span, and survive for decades in the epidermis (Quevedo et al. 1969). However, the longevity of melanocytes and their resistance to apoptosis is a double-edged sword, since these properties make them vulnerable to mutations that arise over the years, particularly due to repetitive sun exposure, and might culminate in melanoma formation in high-risk individuals. These properties also explain the resistance of melanoma to various chemotherapeutic agents and to radiation. Given the significance of melanocytes in protection of the skin from UV-induced skin cancers, it is critical to maintain genomic stability of these cells to ensure their proper function and ability to maintain epidermal homeostasis.

2.2

Factors that Determine Cutaneous Pigmentation

Cutaneous pigmentation is determined by the rate of synthesis of melanins (eumelanin and pheomelanin) by melanocytes, the relative eumelanin and pheomelanin contents, and the rate of transfer of melanin-containing organelles, the melanosomes, from melanocytes to keratinocytes (Pathak et al. 1980). These are the main factors that account for individual differences in skin pigmentation. Melanosomes contain enzymes that are essential for melanin synthesis, namely tyrosinase, the rate-limiting enzyme for melanin synthesis, tyrosinase-related protein (TYRP-1), and dopachrome tautomerase (DCT), also known as tyrosinase-related protein 2 (TRP-2) (reviewed by Hearing 2005). The activity of tyrosinase and the protein levels of these three melanogenic enzymes correlate directly with melanin content of melanocytes (Abdel-Malek et al. 1993; Wakamatsu et al. 2006). Melanosomes also express OA1, a G-protein coupled receptor for L-DOPA, an intermediate in the melanin synthetic pathway, and a substrate for tyrosinase, on their membrane (Hearing 2005). The number of melanocytes does not significantly differ among individuals with different pigmentary phenotypes (Szabo 1954). The difference in pigmentation lies primarily in the rate of melanin synthesis, which is determined by many genes that code for regulatory proteins, including melanogenic enzymes, growth factor receptors, and transcription factors, as well as structural proteins that make up the melanosome. The difference in constitutive pigmentation among individuals is primarily dictated by eumelanin, which correlates directly with the extent of pigmentation (Hennessy et al. 2005; Wakamatsu et al. 2006).

Melanocytes interact with keratinocytes by donating fully melanized (mature) melanosomes (Pathak et al. 1980). Melanocytes are dendritic cells, and their dendrites serve as

conduits for the transport of melanosomes to surrounding keratinocytes. In turn, keratinocytes participate in regulating the transfer of melanosomes by expressing protease-activated receptor 2 (PAR-2), a G-protein coupled receptor that is activated upon proteolytic cleavage by trypsin, or by binding of its agonist, SLIGRL, resulting in increased melanosome phagocytosis in a Rho-dependent manner (Scott et al. 2003; Seiberg et al. 2000). Expression of PAR-2 by keratinocytes is upregulated by UV exposure *in vitro* and *in vivo* (Scott et al. 2001). In the epidermis, the ratio of melanocytes to keratinocytes is 1:34, and the interaction of these cells via transfer of melanosomes has been termed “epidermal melanin unit” (see also Chap. 14). Melanosome transfer is important for normal and uniform skin pigmentation, is increased upon stimulation of melanogenesis, and is critical for optimal photoprotection.

2.3

Pigmentation, the Main Photoprotective Mechanism in the Skin Against Solar UV

Solar UV is the main environmental factor that affects skin pigmentation and the main etiological factor for skin cancers, including melanoma (Epstein 1983; Gilchrest et al. 1999; Pathak 1991). Melanin synthesized by melanocytes is the main photoprotective mechanism in the skin (Gilchrest et al. 1999; Halder and Bridgeman-Shah 1995; Pathak et al. 1980). Melanosomes transferred to keratinocytes form supranuclear caps that protect the nucleus from impinging UV rays (Kobayashi et al. 1998). Also, eumelanin acts as a scavenger of reactive oxygen species produced upon exposure to UV, and thus reduces the oxidative damage to DNA, proteins, and lipids (Bustamante et al. 1993). Melanin in the epidermis is also photoprotective for dermal fibroblasts, preventing photoaging caused by UV, particularly long wavelength UVA (Gilchrest and Rogers 1993). An interesting paradigm is that increased melanin synthesis is part of the DNA damage response of melanocytes, as treatment of human skin with DNA oligonucleotides that are homologous to the telomere 3' overhang sequence (T-oligos) enhanced nucleotide excision repair and subsequently increased epidermal melanin content (Arad et al. 2006).

There is overwhelming clinical and epidemiological evidence for the role of melanin in prevention of sun-induced skin cancers (Epstein 1983; Halder and Bridgeman-Shah 1995; Newton Bishop and Bishop 2005). The incidence of these cancers is by far higher in individuals with fair skin, with low melanin content, than in individuals with dark skin containing high levels of melanin, mainly eumelanin. Experimental evidence shows that exposure to UV results in less DNA photoproducts in dark-skinned individuals with high melanin (mainly eumelanin) content than in light-skinned individuals with low melanin content (Tadokoro et al. 2003). Similarly, an inverse relationship between eumelanin content and the induction of DNA photoproducts was found in cultured human melanocytes derived from donors with different pigmentary phenotypes, with DNA photoproducts being lowest in melanocytes with the highest eumelanin content, and highest in melanocytes that have least eumelanin content (Hauser et al. 2006; Smit et al. 2001).

2.4

Evidence for a Paracrine/Autocrine Network in Human Skin

A symbiotic relationship exists between cutaneous melanocytes, keratinocytes, and fibroblasts. It is well established that a complex and well-regulated paracrine/autocrine network is present in human skin, and that this network is upregulated in response to stress, such as in response to UV or inflammation. In turn, the paracrine/autocrine factors mediate many of the stress responses of epidermal cells. Many of the cytokines and growth factors synthesized by keratinocytes and fibroblasts play important roles in regulating melanocyte function and survival (summarized in Table 2.1, Fig. 2.1). The first evidence for keratinocyte-derived paracrine factors that affect melanocytes came from the observation that medium conditioned by cultured human keratinocytes stimulated the proliferation and melanogenesis of cultured normal human melanocytes (Gordon et al. 1989). Additional evidence came from the observation that melanocytes cocultured with keratinocytes exhibited a dose-dependent increase in melanogenesis following irradiation with very low doses of UVB, while melanocytes in monoculture required irradiation with at least a tenfold higher dose of UVB in order to stimulate melanogenesis (Duval et al. 2001). These latter results implicated keratinocyte-derived factors in the melanogenic response of melanocytes to UVB. Medium conditioned with human fibroblasts also stimulated the proliferation of cultured human melanocytes (Imokawa et al. 1998). Collectively, these results provide evidence for the existence of a paracrine network in human skin that modulates melanocyte function, proliferation, and survival under constitutive conditions and in response to UV.

2.4.1

The Melanocortin 1 Receptor Physiological Agonists and Antagonists are Epidermal-Derived Factors that Regulate Eumelanin and Pheomelanin Synthesis

There has been particular interest in understanding the regulation of eumelanin and pheomelanin synthesis by melanocytes, given the significance of eumelanin in photoprotection, and to elucidate the underlying causes for the diversity of human pigmentation. Stimulation of eumelanin synthesis results mainly from activation of the melanocortin 1 receptor (MC1R), a G_s-protein-coupled receptor expressed on melanocytes (Chhajlani and Wikberg 1992; Hunt et al. 1995; Mountjoy et al. 1992; Suzuki et al. 1996). The physiological agonists for the human MC1R are α -melanocyte stimulating hormone (α -MSH) and adrenocorticotrophic hormone (ACTH), which bind the MC1R with the same affinity (Suzuki et al. 1996). Pro-opiomelanocortin, the precursor for ACTH and α -MSH, is synthesized and processed by cultured keratinocytes and melanocytes, and its expression in the skin is increased by UV exposure (Chakraborty et al. 1996; Corre et al. 2006; Suzuki et al. 2002; Wakamatsu et al. 1997). Actually, the presence of MSH peptides in human skin was reported decades ago, long before a physiological role for these peptides in human pigmentation was identified (Thody et al. 1983). Therefore, MC1R agonists are paracrine/autocrine factors that are expected to participate in the UV response of melanocytes. This

Table 2.1 Epidermal Melanocytes, Regulation of Their Survival, Proliferation, and Function in Human Skin. The following is the list of autocrine/paracrine factors discussed in the text, stating their cellular origin, role in pigmentation, and effects on melanocytes.

Factor	Synthesized by	Role in pigmentation	Melanocyte function
POMC derived: α -MSH, ACTH	KC, MC	↑	MC1R agonist (↑ cAMP)
POMC derived: β -Endorphin	KC, MC	↑	Inhibits cAMP, unknown signaling pathway
Agouti signaling protein (ASIP)	Skin, cell of origin Unknown	↓	MC1R antagonist
Human β defensin 3 (HBD3)	KC	↑ in dog and mouse; unknown in human	MC1R antagonist
Bone morphogenetic protein (BMP-4)	KC, MC	↓	↓ Tyrosinase activity, ↓ TRP-1, ↓ MC1R
Interleukin-1 α/β	KC, MC	↓	↓ Tyrosinase activity, ↓ proliferation
Tumor necrosis factor- α (TNF- α)	KC	↓	↓ Tyrosinase activity, ↓ proliferation
Endothelin-1 (ET-1)	KC	↑	↑ Tyrosinase activity, ↑ proliferation, ↑ survival
Stem cell factor (SCF)	KC, FB	↑	↑ proliferation, ↑ survival, ↑ dendricity
Hepatocyte growth factor (HGF)	KC, FB	↑	↑ proliferation, ↑ migration
Basic fibroblast growth Factor (bFGF)	KC, FB	↑	↑ proliferation
Prostaglandins: PGE ₂ and PGF _{2α}	KC, MC	↑	↑ dendricity, ↑ tyrosinase activity
Leukotrienes: LTC ₄ and LTD ₄	KC	↑	↑ proliferation
Corticotropin releasing hormone	KC, MC	↑	↑ cAMP, ↑ <i>POMC</i> gene (↑ ACTH)
Nerve Growth factor (NGF)	KC	—	↑ dendricity, ↑ migration, ↑ survival
Neurotrophin 3 (NT-3)	KC, FB	—	↑ survival
Semaphorin 7a	KC, FB	—	↑ spreading, ↑ dendricity
Neuregulin-1 (NRG-1)	KC, FB	↑	↑ pigmentation, ↑ dendricity, ↑ MC size
Nitric oxide	KC, MC	↑	↑ Melanogenesis

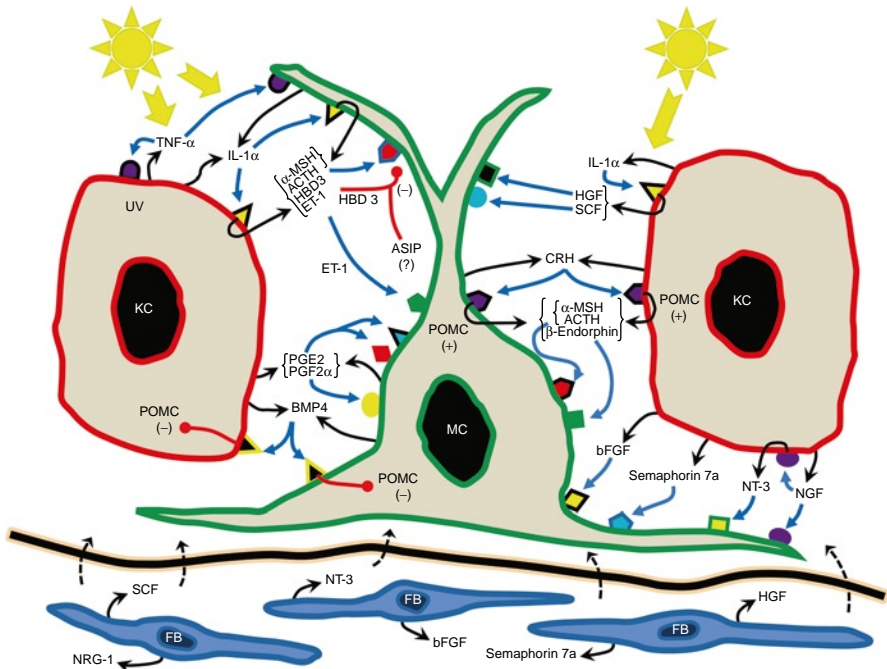


Fig. 2.1 Summary of the major participants in the paracrine/autocrine network that regulates melanocyte function, survival, and proliferation and the regulation of this network by UV. Irradiation of the skin by UV upregulates the expression of the primary cytokines $\text{TNF-}\alpha$ and $\text{IL-1}\alpha$ by keratinocytes, both of which directly affect melanocytes. In turn, $\text{IL-1}\alpha$, which is also synthesized by melanocytes, increases the production of α -MSH and ACTH by keratinocytes and melanocytes, as well as HBD3, ET-1, HGF, and SCF by keratinocytes. In response to UV, CRH production is increased by both keratinocytes and melanocytes. CRH affects melanocytes directly, and indirectly by increasing the expression of POMC, and hence its derivatives α -MSH, ACTH, and β -endorphin. Melanocytes and keratinocytes synthesize BMP-4, which affects melanocytes directly, and might also inhibit POMC production by keratinocytes and melanocytes. Additionally, both keratinocytes and melanocytes synthesize PGE_2 and $\text{PGF}_{2\alpha}$. Keratinocytes synthesize bFGF and semaphorin 7a, and also NGF, which affects melanocytes directly, as well as indirectly by enhancing the production of NT-3 by keratinocytes. Fibroblasts contribute to the paracrine network by synthesizing HGF, SCF, NT-3, semaphorin 7a, and NRG-1

is supported by the observation that activation of the cAMP pathway by α -MSH is critical for UV-induced melanogenesis (i.e., tanning response), and enables UV-irradiated human melanocytes to overcome growth arrest (Im et al. 1998).

Agouti signaling protein (ASIP) is the physiological antagonist of the MC1R that acts as an inverse agonist, competing with α -MSH for receptor binding, and abrogating the activation of the cAMP pathway, the main signaling pathway of the activated MC1R (Suzuki et al. 1997). Concomitant treatment of cultured human melanocytes with α -MSH and ASIP blocked the mitogenic and melanogenic effects of α -MSH. In mice, the

recessive yellow mutation that causes loss of function of the *mc1r* results in a yellow coat color (Robbins et al. 1993). Similarly, overexpression of *agouti* results in the same pigimentary phenotype, due to inhibition of eumelanin synthesis, in addition to other pleiotropic effects, which include obesity, diabetes, and increased susceptibility to develop tumors (Siracusa 1994). Pheomelanin synthesis is considered to be the default pathway, which resumes in the absence of MC1R signaling, unlike eumelanin synthesis that has stringent requirements, including high concentration of tyrosine, the substrate for tyrosinase, and activation of the MC1R, which leads to stimulation of cAMP formation and increase in activities and protein levels of the melanogenic enzymes tyrosinase, TYRP-1 and TRP-2 (Abdel-Malek et al. 1995; Sakai et al. 1997).

Another factor that affects eumelanin/pheomelanin synthesis is human β -defensin 3 (HBD3), an antimicrobial peptide which was cloned from human keratinocytes, and is best known for its role in the innate immunity (Candille et al. 2007; Harder et al. 2001). Genetic studies on dogs revealed that mutation in the *HBD3* gene resulted in black coat color, an effect that was postulated to be due to inhibition of ASIP binding to the MC1R (Candille et al. 2007). Receptor binding assays revealed that HBD3 acts as a competitive inhibitor of α -MSH binding to the MC1R. The role of HBD3 in regulating the ability of human melanocytes to synthesize eumelanin is yet to be determined.

2.4.2

Antagonistic Effects of Bone Morphogenetic Protein and Noggin on Pigmentation, and Their Potential Regulation of MC1R Agonists and Antagonists

Two interesting modulators of melanogenesis are bone morphogenetic protein-4 (BMP-4) and noggin, which modulate melanogenesis, directly, and possibly indirectly by regulating the expression of the MC1R agonists and ASIP. BMP-4 is a member of the TGF- β superfamily, and has been shown to be produced by both human melanocytes and keratinocytes, and to inhibit melanogenesis in human melanocytes (Yaar et al. 2006). The inhibitory effect of BMP-4 involved reduction in the levels of the melanogenic enzymes tyrosinase, and TYRP-1, as well as MC1R (Park et al. 2009; Yaar et al. 2006). The BMP-4 receptors-1A, -1B, and -2 are expressed by human melanocytes, confirming the role of BMP-4 as a paracrine/autocrine factor (Yaar et al. 2006). Irradiation of melanocytes with UV downregulated the expression of BMP-4 receptor-1B, which might be a mechanism by which UV stimulates melanogenesis. Studies on mouse coat color showed that noggin acts as an antagonist of BMP-4, and noggin overexpression reduced the expression of ASIP (Sharov et al. 2005). On the other hand, BMP-4 enhanced the expression of ASIP by primary mouse keratinocytes and fibroblasts. Moreover, in the pituitary gland, BMP-4 repressed the expression of *Pro-opiomelanocortin* (POMC) by corticotrophs (Nudi et al. 2005). In addition to increasing ASIP, potential inhibition of POMC production in the skin, and reduction of MC1R expression in melanocytes might be a mechanism by which BMP-4 inhibits pigmentation, and reversal of these effects by noggin might be a mechanism to stimulate melanogenesis. The effects of BMP-4 and noggin add another layer of complexity to the regulation of melanogenesis upstream of POMC, ASIP, and MC1R.

2.4.3

Identification of the Nature of Paracrine Factors in the Skin

Analysis of the factors in medium conditioned by fibroblasts revealed that they included the cytokines interleukin (IL)-1 α and tumor necrosis factor (TNF)- α , hepatocyte growth factor (HGF), and stem cell factor (SCF) known to be induced by these cytokines, and basic fibroblast growth factor (bFGF) (Imokawa et al. 1998). It has long been known that production of primary cytokines, namely IL-1 α and TNF- α , by keratinocytes is upregulated by UV (Kock et al. 1990; Kupper et al. 1987), and that these cytokines regulate the synthesis of potent mitogenic and melanogenic factors by keratinocytes, such as α -MSH and endothelin-1 (ET-1) (Chakraborty et al. 1996; Imokawa et al. 1992). Human melanocytes were also found to synthesize IL-1 α and β , and to respond to IL-1 α and TNF- α with inhibition of proliferation and melanogenesis, suggesting that these cytokines directly modulate melanocyte function and proliferation (Swope et al. 1991; Swope et al. 1994). Recently, using whole human genome microarray analysis, it was shown that repetitive irradiation of human skin *in situ* by UVB resulted in altered expression of genes that encode for paracrine factors or their receptors (Choi et al. 2010a). These genes included those that encode for HGF, bFGF, IL-1 α and β , and GM-CSF, and for the receptors PAR-2, the SCF receptor Kit, the endothelin-1 B receptor (ETBR), and MC1R. These *in vitro* and *in vivo* findings provide compelling evidence that many factors that regulate melanocytes are synthesized locally in the skin.

The first keratinocyte-derived paracrine factor for melanocytes to be identified was bFGF, an essential mitogen for melanocytes (Halaban et al. 1988). Basic FGF stimulates melanocyte proliferation by binding and activating a specific tyrosine kinase receptor (Pittelkow and Shipley 1989). Another important keratinocyte-derived paracrine factor is ET-1, which induces melanocyte proliferation, melanogenesis, and migration (Horikawa et al. 1995; Tada et al. 1998; Yada et al. 1991; Yohn et al. 1993). Human melanocytes predominantly express ETBR, a G_q-coupled receptor, which when bound by either the ET-1 or ET-3, activates PKC, intracellular calcium mobilization, and nonreceptor tyrosine kinases (Imokawa et al. 1992; Tada et al. 1998). Mutations in either the gene for endothelin-3 (which during embryonic development, mimics ET-1 in its effects and mechanism of action) or ETBR result in Hirschprung's disease Type II, which is characterized by hypopigmentation due to death of melanoblasts during their migratory route from the embryonic neural crest, and by aganglionic megacolon due to absence of neural crest-derived ganglia (Puffenberger et al. 1994). Treatment of cultured human keratinocytes with IL-1 α or irradiation of human skin *in vivo* induced the production of ET-1 (Imokawa et al. 1992). Treatment of cultured human melanocytes with ET-1 upregulated the expression of the ETBR, an effect that is expected to sustain the responsiveness of melanocytes to this factor (Tada et al. 1998).

Two important paracrine factors are stem cell factor (SCF) and hepatocyte growth factor (HGF), both of which are synthesized by both keratinocytes and fibroblasts (Imokawa et al. 1998; Matsumoto et al. 1991). Stem cell factor elicits its mitogenic and survival effects on melanocytes by activating a specific tyrosine kinase receptor, c-kit, and mutations in the Kit gene result in piebaldism, which is characterized by depigmented skin patches as a consequence of death of melanocytes during their migratory route during embryonic development to populate the epidermis (Giebel and Spritz 1991). In adult skin, SCF is required for melanocyte maintenance, since injection of c-kit antibody resulted in loss of pigmentation due to melanocyte death (Grichnik et al. 1998). Hepatocyte growth

factor, which activates the tyrosine kinase receptor c-Met, is an important factor for melanocyte homing to the epidermis during embryonic development and for stimulating human melanocyte proliferation (Matsumoto et al. 1991). The observation that HGF transgenic mice have extensive skin melanosis provides genetic evidence for the significance of HGF in directing the migration of melanocytes to the epidermis (Otsuka et al. 1998).

2.4.4

Eicosanoids as Paracrine/Autocrine Factors for Melanocytes

The eicosanoids, prostaglandins (PGs), and leukotrienes (LTs) are lipid signaling intermediates that are derived from arachidonic acid via the cyclooxygenase and lipoxygenase pathway, respectively. Although the major source of PGs in the skin is the keratinocytes, experimental evidence showed that melanocytes also synthesize the major two forms of PGs, PGE_2 and $\text{PGF}_{2\alpha}$, in response to UV irradiation (Scott et al. 2005). Human melanocytes express cyclooxygenase (COX)-1 and -2, the latter of which is the inducible form, and the rate-limiting enzyme for the synthesis of PGs (Nicolaou et al. 2004). Human melanocytes express FP receptor, the receptor for $\text{PGF}_{2\alpha}$ *in vitro* and *in vivo*, and expression of this receptor was upregulated upon UV exposure (Scott et al. 2005; Starner et al. 2010). Melanocytes responded to $\text{PGF}_{2\alpha}$ with stimulation of dendricity, and melanogenesis that was evidenced by increased activity and protein levels of tyrosinase. Multiple irradiations of cultured melanocytes with moderate noncytotoxic doses of UV induced the synthesis of PGE_2 via activation of cytoplasmic phospholipase A_2 (cPLA $_2$), the rate-limiting enzyme in eicosanoid synthesis (Starner et al. 2010). Melanocytes expressed two of the four PGE_2 G-protein coupled receptors, EP2 and EP4, and treatment of human melanocytes with PGE_2 increased cAMP formation, and stimulated proliferation and tyrosinase activity (Starner et al. 2010). The leukotrienes LTC_4 and D_4 were found to be potent mitogens for cultured human melanocytes (Morelli et al. 1989). These results demonstrate the participation of eicosanoids in the autocrine/paracrine network that regulates melanocyte proliferation and function and the response to UV via activation of specific PG and LT receptors.

2.4.5

An Equivalent of the Hypothalamic/Pituitary/Adrenal Axis Is Present in Human Skin

Studies from various laboratories provided evidence that the skin is a “neuroendocrine organ,” which contains an equivalent of the systemic stress-induced hypothalamic/pituitary/adrenal axis. Skin cells, including melanocytes, express corticotropin releasing hormone (CRH) mRNA and protein, and also its receptor CRH-R1 (Funasaka et al. 1999; Slominski 1998; Slominski et al. 1995). Treatment of human melanocytes with CRH resulted in increased cAMP levels, and upregulated the expression of *POMC* gene. The latter effect led to increased production of the POMC derivative ACTH, which contains within its structure the entire amino acid sequence of α -MSH. These two bioactive peptides are produced upon processing of POMC by the enzymes proconvertase 1 and 2, respectively. The significance of POMC in human pigmentation was supported by the observation that mutations in the human *POMC* gene that affected its expression resulted

in red hair phenotype in addition to metabolic abnormalities, such as adrenal insufficiency and obesity (Krude et al. 1998). In melanocytes, ACTH induced the production of cortisol and corticosterone, which feedback negatively to inhibit the production of ACTH, and thus terminate the response to stress (Slominski et al. 2005). These series of events recapitulate the functional hierarchy in the hypothalamic/pituitary/adrenal axis, and provide further evidence for the participation of the melanocyte in the cutaneous stress response, by producing and responding to stress-induced factors.

In addition to α -MSH and ACTH, β -endorphin, a third bioactive peptide derived from POMC, is produced *in vitro* and *in situ* by keratinocytes and melanocytes upon the cleavage of β -lipotropic hormone (β -LPH) (Kausar et al. 2003; Slominski 1998). Production of β -endorphin by melanocytes correlated with their differentiation status, as determined by their dendricity and pigmentation, indicating that β -endorphin functions as an autocrine factor (Kausar et al. 2003). Melanocytes and keratinocytes express functional μ -opiate receptors, and treatment of cultured human melanocytes with β -endorphin stimulated proliferation, dendricity, and melanogenesis (Kausar et al. 2003). In melanocytes, both β -endorphin and μ -opiate receptors colocalized in premelanosomes and stage II melanosomes, suggesting that the ligand and receptor internalize into immature melanosomes where they induce melanogenesis. Unlike melanocortins whose effects are mediated by activating the cAMP pathway, β -endorphin inhibited adenylate cyclase, thus reducing cAMP levels. The signaling pathway(s) that mediates the effects of β -endorphin on melanocytes is yet to be determined. The increase in β -endorphin production in response to UV might explain the “addictive” behavior of sun worshippers, and the feeling of wellness associated with sun exposure.

2.4.6

Neurotrophins as Members of the Cutaneous Neuroendocrine Paracrine Network

Given that the cutaneous epidermis and the nervous system arise from a common ectodermal origin, it was postulated that growth factors that regulate the survival and function of neurons play a role in the maintenance of epidermal homeostasis. Neurotrophins (NTs) are a family of neuronal growth factors comprised of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, and NT-4, two of which, namely NGF and NT-3, are synthesized in the skin (reviewed by Botchkarev et al. 2006). Human keratinocytes, particularly basal keratinocytes with the highest proliferation capacity, were found to synthesize and release NGF, which in turn enhanced the secretion of NT-3, which was also synthesized by dermal fibroblasts (Marconi et al. 2003; Yaar et al. 1994). Neurotrophins share about 50% amino acid sequence homology, and interact with high-affinity tyrosine kinase receptors. Tyrosine kinase receptors (Trk) A, B, and C are the high-affinity receptors for NGF, BDNF, and NT-3, respectively. All NTs interact with the low-affinity p75 NT receptor, a member of the TNF- α family of receptors. Depending on the intracellular adaptor molecules that interact with p75 receptor, its signaling may be linked to the JNK-p53-Bax pro-apoptotic pathway, or to the NF- κ B survival pathway. Human melanocytes responded to NGF with increased migration and dendricity, and additionally, with inhibition of apoptosis after UV irradiation via increasing the levels of the antiapoptotic Bcl2

(Stefanato et al. 2003; Yaar et al. 1991; Zhai et al. 1996). Both NGF and NT-3 increase the survival of human melanocytes maintained in growth factor-depleted culture medium (Yaar et al. 1994; Zhai et al. 1996). Human melanocytes expressed p75^{NT} receptor, and this was increased upon UV irradiation (Yaar et al. 1994). Melanocytes also express low levels of TrkC, the receptor for NT-3, and TrkA expression was induced upon stimulation of protein kinase C. Based on these studies, it was concluded that NGF, which is the most prevalent NT that is constitutively produced by keratinocytes, might ensure the survival of melanocytes by inhibiting UV-induced apoptosis, and NT-3 that is strongly expressed in fibroblasts might also participate in melanocyte maintenance.

2.4.7

Semaphorin 7 a, A Neuronal Factor Synthesized in Human Skin

Semaphorin 7 is a member of the secreted and membrane-bound semaphorin family of proteins that function in neuronal pathfinding and axonal guidance (Yazdani and Terman 2006). It was the first glycosylphosphatidylinositol-linked semaphorin to be identified, and shown to bind plexin C1 and β 1-integrin receptor (Pasterkamp et al. 2003; Sato and Takahashi 1998; Tamagnone et al. 1999). Semaphorin 7a was found to be expressed in the skin *in vivo*, and in keratinocytes and fibroblasts, as demonstrated *in vitro* (Scott et al. 2008). In response to UV, fibroblasts exhibited a marked increase in semaphorin 7a expression. The role of semaphorin 7a as a paracrine factor for melanocytes was demonstrated by the finding that treatment of cultured human melanocytes with exogenous semaphorin 7 a, or coculturing of melanocytes with cells expressing semaphorin 7a led to increased spreading and dendricity. These effects were mediated by binding the β 1- integrin receptor, and were inhibited by plexin C1. These results identify a novel neuronal factor expressed in the skin, which regulates melanocyte spreading and dendricity positively via β 1-integrin receptor, or negatively by interacting with Plexin C1. These effects might have significant implications on melanosome transfer, an important determinant of cutaneous pigmentation.

2.4.8

Neuregulin-1, A Newly Identified Neuroendocrine Factor Synthesized by Human Epidermal and Dermal Cells

A novel neuroendocrine factor Neuregulin-1 (NRG-1) was recently identified as a fibroblast-derived factor that regulates constitutive human pigmentation (Choi et al. 2010b). Neuregulin-1 is a secreted growth factor that is expressed in the central nervous system and is critical for neuronal differentiation, migration, and dendrite formation (Krivosheya et al. 2008). Cultured fibroblasts derived from skin type VI donors expressed higher levels of NRG-1 than fibroblasts derived from skin type II donors. *In vivo*, NRG-1 was highly expressed in the epidermis as well as the dermis of skin type VI donors, but was expressed at very low levels only in the dermis of skin type II donors. Treatment with exogenous NRG-1 gave rise to increase in pigmentation, melanocyte size, and dendricity in cultured skin substitutes, and these effects were more prominent in skin substitutes representative of

skin type VI than in their counterparts representative of skin types IV or II. The effects of NRG-1 were mediated by binding to ErbB3 and ErbB4 receptors, tyrosine kinase receptors that belong to the family of epidermal growth factor receptors. Interestingly, ErbB3 expression was higher in melanocytes derived from dark skin than in melanocytes cultured from light skin, suggesting that activation of this receptor is responsible for the melanogenic effects of NRG-1. On the other hand, ErbB4 expression was expressed at higher levels in melanocytes derived from light skin, compared to melanocytes from dark skin. These novel findings implicate NRG-1 and its receptors in regulating constitutive pigmentation.

2.5

Role of Melanocyte and Keratinocyte-Derived Nitric Oxide in Regulating Pigmentation

In addition to enhancing the production of a large panel of paracrine and autocrine growth factors, UV stimulated the production of nitric oxide (NO) by both keratinocytes and melanocytes (Romero-Graillet et al. 1996; Romero-Graillet et al. 1997). In keratinocytes, this effect was mediated by increased constitutive NO synthase (Romero-Graillet et al. 1997), and might involve the activation of Akt, as was shown for endothelial NO synthase (Dimmeler et al. 1999). The melanogenic effect of UV was abrogated to a large extent upon treatment with NO scavengers, while treatment of melanocytes with exogenous NO donors stimulated melanogenesis and dendricity, lending direct evidence for the melanogenic effect of NO (Romero-Graillet et al. 1997). This effect was mediated by increasing the levels of cGMP, and treatment of melanocytes with guanylate cyclase inhibitors blocked the UV-induced melanogenesis (Romero-Graillet et al. 1996). It is possible that the melanogenic effect of cGMP is indirect, resulting from inhibition of cAMP phosphodiesterase, which leads to increased cAMP levels, the principle mechanism for stimulation of melanogenesis. In B16 and human melanoma cells, α -MSH increased the UV-induced NO levels, and as in normal human melanocytes, NO stimulated melanogenesis (Tsatmali et al. 2000). This melanogenic effect was abrogated by inhibition of the inducible NO synthase. The findings that α -MSH modulate the production of NO raises the question whether NO functions as an autocrine factor or as second messenger that mediates the effects of α -MSH.

2.6

Signaling Pathways Responsible for Regulating Melanocyte Proliferation, Survival, and Function

The first melanocyte growth medium was based on the use of phorbol esters that activate protein kinase C, and cAMP inducers, such as cholera toxin (Eisinger and Marko 1982). This underscored the significance of these signaling pathways in sustaining the survival, proliferation, and function of human melanocytes. Later, it was shown that paracrine growth factors for melanocytes that activate tyrosine kinase receptors, such as bFGF, SCF, and HGF, as well as ET-1 that activates Gq coupled receptor, can substitute for phorbol esters in

the culture medium, and stimulate proliferation by activating the MAP kinases ERK1/2 (Bohm et al. 1995; Swope et al. 1995; Tada et al. 1998). Activation of ERK1/2 resulted in phosphorylation, hence activation of the transcription factor cyclic AMP response element binding protein (CREB), upstream from the melanocyte master regulator, the transcription factor Mitf (Bohm et al. 1995; Tada et al. 2002). Signaling pathways that were involved in ERK1/2 activation included PKC, tyrosine kinases, and intracellular calcium mobilization. The cAMP pathway has long been known to be essential for stimulating melanogenesis in pigment cells (Hirobe and Takeuchi 1977; Pawelek et al. 1973). The main signaling pathway for α -MSH is cAMP, and α -MSH is primarily a melanogenic factor for human melanocytes (Abdel-Malek et al. 1992). In contrast to other growth factors, such as bFGF or ET-1, α -MSH, and other cAMP inducers are poor activators of the ERK1/2 pathway, yet α -MSH interacts synergistically with factors that stimulate PKC, tyrosine kinases, or intracellular calcium mobilization to activate these MAP kinases and their downstream targets, leading to increased melanocyte proliferation and melanogenesis (Herraiz et al. 2011; Tada et al. 2002).

The transcription factor Mitf is a member of the basic helix-loop-helix leucine zipper transcription factors, and is considered the “master regulator” in melanocytes (reviewed by Steingrimsson et al. 2004) (see also Chap. 5). Germline mutations in *Mitf* result in Waardenburg Syndrome type 2, characterized by congenital white forelock, sensorineural deafness, and asymmetric iris color. The significance of Mitf lies in its ability to regulate the expression of many target genes that are involved in melanin synthesis, proliferation, and survival of melanocytes (reviewed by Cheli et al. 2009). Direct targets for Mitf are the genes for the melanogenic enzymes Tyrosinase, *TYRP1*, *DCT*, and the melanosomal genes PMel 17, OA1, as well as Rab27A that is involved in melanosome transfer. Other Mitf target genes are Bcl2, BIRC7, and DICER that are involved in regulating melanocyte survival (see also Chaps. Apoptosis and miRNA), KIT, NGFR, and ETBR genes that encode for receptors for melanocyte growth and survival factors (SCF, NGF, and ET-1, respectively), and APE/Ref1 that regulates the redox state, as well as HIF1 α , which is induced by hypoxia. Additional targets for Mitf include the cell cycle regulatory genes TBX2 and CDK2, as well as CDKN1A (p21) and CDKN2A.

It is well established that Mitf is regulated by the ERK1/2 pathway. Treatment of melanoma cells with SCF activated ERK, which resulted in the phosphorylation of Mitf on Ser 73 and Ser 409 (Wu et al. 2000). Phosphorylation of Mitf transiently increased its transcriptional activity, as evidenced by increasing tyrosinase expression upon phosphorylation of Mitf on Ser 73 by SCF, and subsequently targeting Mitf for ubiquitination (Hemesath et al. 1998; Wu et al. 2000). Activation of the cAMP pathway in mouse and human melanoma cells by α -MSH or forskolin also resulted in the phosphorylation of Mitf (Price et al. 1998). Treatment of cultured human melanocytes with α -MSH and/or ET-1 in the presence of bFGF increased total protein levels of Mitf, as well as its phosphorylated form (Kadekaro et al. 2005). The observed increase in Mitf protein was also regulated transcriptionally, as global gene analysis of melanocytes treated with α -MSH revealed increased *Mitf* gene expression (Kadekaro et al. 2010).

Exposure to UV activated the stress MAP kinases p38 and JNK/SAPK, which regulate the activity of downstream transcription factors that mediate the stress response (Ono and Han 2000; Rosette and Karin 1996). Increase in pigmentation, i.e., tanning, is considered part of the stress response, which is mediated by the paracrine/autocrine network that is activated by UV. The transcription factor Upstream Stimulating Factor-1 (USF-1) was activated by p38 and proved to be an important regulator of *MC1R* and *POMC* expression

in melanocytes exposed to UV (Corre et al. 2004). In addition, USF-1 upregulated the expression of Tyrosinase, *TYRP-1*, and *DCT*. Phosphorylation of USF-1 resulted in its activation and enhanced its ability to bind DNA (Galibert et al. 1997). Another transcription factor, ATF2, known to regulate genes involved in DNA repair, such as *XPC* and *ERCC1*, apoptosis, such as *Bcl2*, and the cell cycle, such as *CDK4*, was also activated by p38, as well as by JNK (Fuchs et al. 2000; Hayakawa et al. 2004), and thus is expected to play an important role in the DNA damage response of melanocytes to UV. Global gene analysis of human melanocytes irradiated with UV or treated with α -MSH revealed that ATF2 and its target genes *Bcl2*, *CDK4*, and *ERCC1* were reduced in expression by UV, and upregulated in expression by α -MSH (Kadekaro et al. 2010). The modulation of ATF2 and *Bcl2* expression by UV and α -MSH was confirmed by Western blotting, indicating that ATF2 participates in the UV response of human melanocytes.

The p53 transcription factor is considered a universal sensor of genotoxic stress (Chouinard et al. 2002; Huang et al. 1999; Liu et al. 1996). It is known to accumulate following UV exposure due to its stabilization, and induces growth arrest in order to allow for DNA repair; this was shown to occur in human melanocytes (Liu et al. 1996; Marrot et al. 2005; Medrano et al. 1995). P53 was regulated by p38, and in turn it regulated the expression of Tyrosinase and *TYRP-1* (Chouinard et al. 2002; Khlgatian et al. 2002; Nylander et al. 2000). In mouse skin, p53 was found to upregulate the expression of POMC, the precursor for melanocortins and β -endorphin that stimulate melanogenesis (Cui et al. 2007). Another study found that mutations in the ribosomal protein s (Rps) genes, *Rps6*, *Rps19*, and *Rps20* gave rise to dark skin, due to epidermal melanocytosis (McGowan et al. 2008). The dark skin phenotype is dependent on increased p53 in keratinocytes, which led to increased expression of *SCF* that encodes for a potent mitogen and melanogenic factor for melanocytes. These intriguing results observed in mouse skin implicate p53 in regulating the expression of important paracrine factors known to have significant impact on melanocytes. Recently, a positive feedback loop between p53, SCF, and ET-1 was described (Murase et al. 2009). Inducing high levels of p53 in cultured human keratinocytes resulted in increased production of SCF and ET-1, and treatment of cultured human melanocytes with either SCF or ET-1 increased phosphorylation of p53 on Ser 15, which led to its stabilization. In contrast, silencing or inhibition of p53 in melanocytes resulted in decreased Kit expression, inhibition of Mitf, as well as reduced tyrosinase levels and melanin content. In cultured skin substitutes, inhibition of p53 suppressed melanogenesis and led to reduced pigmentation, and silencing of p53 *in vivo* inhibited pigmentation of UV-irradiated mouse ears. These effects of p53 confirm its significance in regulating pigmentation, particularly the tanning response to UV.

2.7

The *MC1R*, a Main Determinant of the Diversity of Human Pigmentation, and a Melanoma Susceptibility Gene

The *MC1R* is a highly polymorphic gene, with more than 75 allelic variants expressed in different human populations (reviewed by Garcia-Borron et al. 2005). The *MC1R* is considered a main determinant of the diversity of human pigmentation, with the wild-type

MC1R predominantly expressed in Africa, where high eumelanin content in the skin is critical for optimal photoprotection. A few of the *MC1R* variants, mainly R151C, R160W and D294H, are strongly associated with red hair phenotype (Box et al. 1997; Smith et al. 1998). Expression of any two of these variants in the homozygous or compound heterozygous state results in loss of function of the receptor, disrupting its ability to signal when bound by its agonists (Kadekaro et al. 2010; Scott et al. 2002). Epidemiological studies from different populations in different geographical locations have demonstrated that these allelic variants are also associated with poor tanning ability and increased risk for melanoma and nonmelanoma skin cancer (Box et al. 2001; Kennedy et al. 2001; Palmer et al. 2000). Currently, the *MC1R* gene is considered a low penetrance melanoma susceptibility gene. However, coexpression of one of the *MC1R* red hair-associated variants with a mutation in the highly penetrant p16 gene significantly increases the risk for melanoma above that caused by the p16 mutation alone (Demenais et al. 2010). Stimulation of eumelanin synthesis by activation of the MC1R confers photoprotection; however, the effect of *MC1R* genotype on melanoma risk is independent of the effect on pigmentation, suggesting that MC1R determines the risk for melanoma by other mechanisms (Kennedy et al. 2001; Landi et al. 2005; Palmer et al. 2000; Stratigos et al. 2006).

2.8

Role of ET-1 and Melanocortins in the DNA Damage Response of Melanocytes

In 2005, novel roles for melanocortins and ET-1 were discovered. In addition to the well-known effects of these factors on melanogenesis and proliferation, they increased the survival of UV-irradiated human melanocytes, enhanced nucleotide excision repair, and reduced oxidative damage by inhibiting the generation of hydrogen peroxide (Kadekaro et al. 2005) (Fig. 2.2). Enhancement of nucleotide excision repair capacity by α -MSH was observed by others, and treatment of human melanocytes with the potent α -MSH analog NDP-MSH increased the transcription of NR4A subfamily of orphan nuclear receptors, which played a role in the MC1R-mediated repair of DNA photoproducts (Bohm et al. 2005; Smith et al. 2008). Treatment of cultured human melanocytes with α -MSH reduced the induction of 8-oxo-guanosine, thus confirming reduction of oxidative DNA damage (Song et al. 2009). The survival effects of α -MSH and ET-1 on UV-irradiated human melanocytes were mediated by activating the MC1R and ETBR, respectively, and were independent of increased melanogenesis, since they were observed in tyrosinase-negative albino melanocytes (Kadekaro et al. 2005). These survival effects were mediated by activating Akt and Mitf, and maintaining high levels of Bcl2, a known Mitf target (Kadekaro et al. 2005; McGill et al. 2002). The recent finding that Mitf activation by α -MSH upregulated the expression of DICER, a RNase II endonuclease that digests premature miRNA to yield mature miRNA, reduced the expression of the proapoptotic BIM (Levy et al. 2010), suggested that decreased BIM expression is involved in the survival effects of α -MSH and ET-1.

The effects of α -MSH on nucleotide excision repair, generation of reactive oxygen species, and oxidative DNA damage received considerable attention, given the high polymorphism of the *MC1R* that result in the differential response of melanocytes derived from

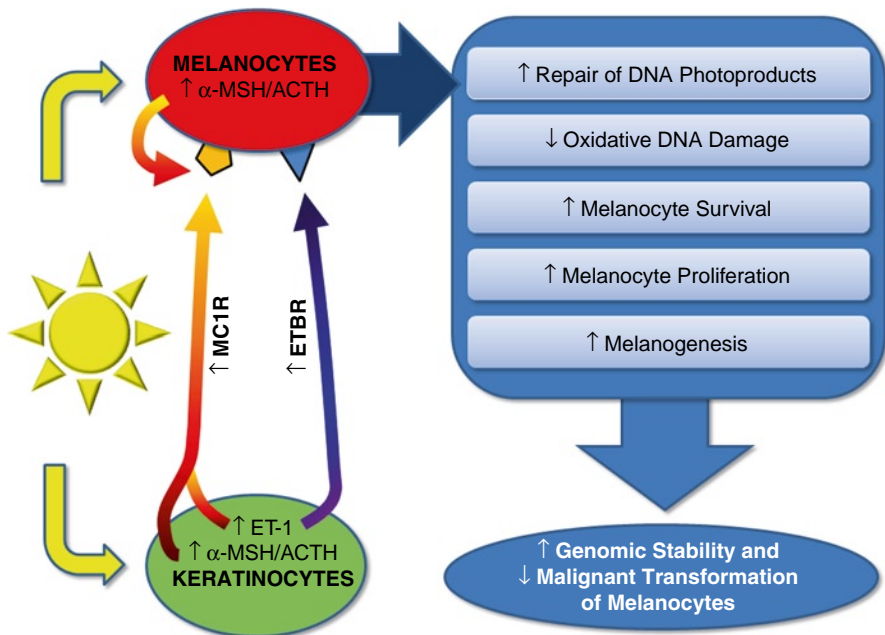


Fig. 2.2 The global effects of α -MSH and ACTH, and ET-1 on melanocytes and their response to UV exposure. Exposure to UV increases the production of ET-1 by keratinocytes, and α -MSH and ACTH by keratinocytes and melanocytes. The melanocortins α -MSH and ACTH, as well as ET-1, upregulate the expression of the MC1R, and ET-1 increases the expression of ETBR on melanocytes. Activation of MC1R and ETBR results in enhanced repair of DNA photoproducts, reduction in generation of reactive oxygen species that lead to oxidative DNA damage, increased melanocyte survival and proliferation, as well as melanogenesis. The effects of these factors on UV-induced DNA damage are expected to maintain genomic stability of melanocytes and reduce the chance for malignant transformation to melanoma

donors with different *MC1R* genotypes to α -MSH and UV (Kadekaro et al. 2010; Scott et al. 2002). These effects required functional MC1R as they were absent in melanocytes that express loss of function receptors. The cAMP pathway mediated the effects of α -MSH on DNA damage, since these effects were also induced by forskolin, a direct activator of adenylate cyclase. Further evidence for the significance of MC1R in reducing the burden of UV-induced DNA damage was provided by the finding that transfection of melanocytes expressing loss-of-function MC1R with the wild-type gene restored the ability to respond to α -MSH by enhanced repair of DNA photoproducts, reduced generation of reactive oxygen species, and increased survival (Kadekaro et al. 2010). These findings provide a molecular mechanism for the increased melanoma susceptibility associated with expression of loss-of-function variants of the *MC1R*, as melanocytes expressing these variants have compromised DNA repair capacity and sustained oxidative stress.

Oxidative DNA damage seems to play an important role in melanocyte transformation to melanoma. Unlike basal or squamous cell carcinomas that originate from keratinocytes, melanoma tumors rarely have UV signature mutations that result from unrepaired

pyrimidine dimers, as in the *p53* gene (Brash et al. 1991; Lubbe et al. 1994). This suggests that other forms of DNA damage are causative for melanoma. Treatment with α -MSH immediately (within minutes) reduced the generation of hydrogen peroxide in UV-irradiated melanocytes (Kadekaro et al. 2005; Kadekaro et al. 2010; Song et al. 2009). This effect absolutely required functional MC1R, was inhibited by ASIP, and was absent in melanocytes that express loss-of-function receptor. Additionally, α -MSH increased the activity of catalase, a first-line-of-defense antioxidant enzyme, and counteracted the inhibitory effect of UV on catalase activity and protein levels in melanocytes expressing functional MC1R (Song et al. 2009). Recently, it was reported that catalase was transported with melanosomes to keratinocytes (Maresca et al. 2010). This intriguing finding suggested that melanocytes protect keratinocytes not only by transferring melanin contained within melanosomes that reduce the penetration of UV rays to nuclear DNA, but also by providing additional catalase, which reduces reactive oxygen species, and prevents oxidative damage. Besides increasing the activity and levels of catalase, α -MSH also upregulated the protein levels of ferritin, an iron sequestrant in melanocytes (Song et al. 2009). Treatment with α -MSH also activated the transcription factor Nrf-2, which regulates the expression of phase II detoxifying enzymes that contain antioxidant response element (ARE) in their promoter (Kokot et al. 2009). Examples of such enzymes are hemeoxygenase-1 (HO-1), γ -glutamylcysteine-synthase, and γ -glutathione S-transferase, which were upregulated by α -MSH in UV-irradiated melanocytes (Kokot et al. 2009).

Microarray analysis of genes altered in expression by α -MSH and/or UV in melanocytes expressing functional versus nonfunctional MC1R revealed that the former responded to α -MSH by altered expression of many genes, particularly those that regulate melanogenesis (e.g., melanogenic enzymes, melanosome biogenesis, transcription factors, growth factor receptors), survival, cell cycle, DNA repair, and oxidative stress, while the latter showed no changes in gene expression, further confirming the refractoriness of these cells to α -MSH (Kadekaro et al. 2010). In general, α -MSH upregulated, while UV downregulated transcription. Importantly, α -MSH reversed the effects of UV on many genes, including some that are involved in the damage response to UV, particularly melanogenesis, DNA repair, cell cycle, oxidative stress, and apoptosis. These effects of α -MSH were only evident in melanocytes expressing functional MC1R, and were absent in melanocytes expressing loss-of-function receptor, lending further explanation for why certain *MC1R* genotypes that cause loss of function of MC1R are associated with increased susceptibility to mutagenesis and melanoma formation.

2.9

Melanocortin Analogs for Melanoma Prevention

Since decades, there has been interest in targeting the MC1R in a strategy to prevent melanoma, as well as nonmelanoma skin cancers. This strategy was initially based on utilizing potent melanocortin analogs for sunless safe tanning that is photoprotective. Injecting human subjects with the potent and best known α -MSH analog NDP-MSH was found to be effective in inducing tanning in the absence of sun exposure (Levine et al. 1991).

Recently, this analog was found to reduce the induction of DNA photoproducts in sun-exposed human skin (Barnetson et al. 2006). However, despite the effectiveness of NDP-MSH, it is not specific to the MC1R, as it can bind the other melanocortin receptors, MC3, 4, and 5R. For the goal of developing small analogs of α -MSH for topical application to prevent skin cancers, tetrapeptide analogs of α -MSH were developed, and shown to surpass α -MSH in their potency to stimulate melanogenesis, and to reduce UV-induced DNA damage and apoptosis (Abdel-Malek et al. 2006). More recently, tripeptide analogs were developed and shown to be capable of activating the MC1R and reducing UV-induced DNA damage (Abdel-Malek et al. 2009). Some of these peptides proved to be highly selective for the MC1R, which should alleviate any side effects that might arise due to binding and activating other MCRs, and two that has been tested so far could permeate human skin, suggesting their possible efficacy in a topical application (Abdel-Malek et al., unpublished data). These analogs require functional MC1R, and are expected to confer photoprotection for individuals with wild-type MC1R, are heterozygous for MC1R variants that reduce receptor function, or mutant for other melanoma susceptibility genes, such as the highly penetrant p16, or PTEN (Demenais et al. 2010; Sosman and Margolin 2009). Others have proposed the use of forskolin, an activator of adenylate cyclase, for melanoma prevention (D'Orazio et al. 2006). Since forskolin, like α -MSH, activates the cAMP pathway, it has similar photoprotective effects as α -MSH (Kadekaro et al. 2010). However, forskolin is nonspecific, and its target, adenylate cyclase, is ubiquitously expressed in all cell types, which precludes its selective use for photoprotection.

2.10

What Normal Melanocytes Teach About Melanoma

Normally, melanocytes in the skin are quiescent, and their homeostasis is maintained through its interaction with keratinocytes and with their microenvironment. During the early stages of melanomagenesis, melanocytes acquire the ability to proliferate and escape from cell cycle regulation by uncoupling from keratinocytes (reviewed by Haass and Herlyn 2005). This is achieved by downregulating the expression of the adhesion molecules E-cadherin, P-cadherin, and desmoglein in response to the paracrine factor HGF via binding to c-Met and activation of ERK1/2 and IP3 kinase (Li et al. 2001). Similarly, ET-1 can downregulate E-cadherin (Jamal and Schneider 2002). Further studies showed that overexpression of bFGF in a human xenograft model followed by UVB irradiation gave rise to hyperplastic melanocytic cells with high-grade atypia, reminiscent of lentiginous melanoma (Berking et al. 2001). Overexpression of bFGF concomitantly with ET-3 and SCF, followed by UVB exposure led to the formation of nests of atypical melanocytes representing melanoma *in situ*, some of which progressed into invasive melanoma (Berking et al. 2004). Given that these paracrine factors are upregulated in expression by UV, an etiological factor for melanoma, it is plausible that sun exposure might lead *in vivo* to deregulation of their expression, or expression of their receptors, e.g., ETBR (Demunter et al. 2001), resulting in uncontrolled melanocyte proliferation.

The MAP kinase ERK1/2 and IP3 kinase pathways are important regulators of melanocyte homeostasis. Mutations that disrupt these pathways, such as the BRAF^{V600E} mutation

that causes constitutive activation of BRAF, upstream of ERK1/2, and loss of PTEN that causes continuous activation of AKT, thus increased proliferation and survival, are common in melanoma (Davies et al. 2002; Sosman and Margolin 2009). Therefore, understanding the regulation of normal melanocytes provides insight into the pathways that lead to melanoma formation, and the opportunity to target components of these pathways for melanoma therapy, as in the currently used BRAF inhibitors that hold promise for melanoma treatment.

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Abstract Melanoma is steadily increasing in incidence while mortality is steady or only increasing slightly. Patterns of incidence, including anatomic site, differ by age and sex as well as geographic location. Light pigmentation and multiple nevi are critical risk factors for the development of melanoma as are family history and intermittent sun exposure. Tanning lamp exposure has been clearly identified as adverse risk for melanoma and other environmental factors are under new evaluation: pesticides, arsenic, polychlorinated biphenyls. Genetic factors modify response to environmental factors, and these are currently being uncovered.

3.1 Introduction

The incidence of cutaneous melanoma is steadily increasing, mainly in populations of European origin and is thus an important public health issue throughout the world. This chapter reviews these trends and suggests a perspective. The chapter covers worldwide incidence patterns, the relationship of host characteristics to incidence, and the relationship of environmental factors to risk.

3.2 Rates and Trends

The current incidence rates for melanoma have continued to increase since 1960 and are highest among the developed countries with some potential increases among developing countries, pointing to either a change in behavior, a change in screening, or a combination of both. Among non-Caucasian populations incidence rates are quite variable and relatively low. World rates vary between a low of 0.2 per 100,000 among females in India to

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55.8 per 100,000 among males in Queensland, Australia. Note that rates in this chapter are standardized to the world population, which is generally younger than the populations in developed countries, and so when evaluating rates, it is important to understand the population to which they are standardized. Thus, for the same time period, the Queensland Cancer Council cites melanoma rates in Queensland among males as 76.4, but this is standardized to the Australian population.

Melanoma is notable for higher rates among non-Hispanic whites and this is noted in the USA SEER registries, where the rates for white males are 19.4/100,000 and white females 14.4/100,000 compared to Hispanic white rates for white males of 3.0/100,000 and 3.2/100,000 for white females (see Figs. 3.1 and 3.2).

Several recent analyses have reported on increasing incidence rates among Hispanics in California and Florida (Cockburn et al. 2006; Rouhani et al. 2010). However, although

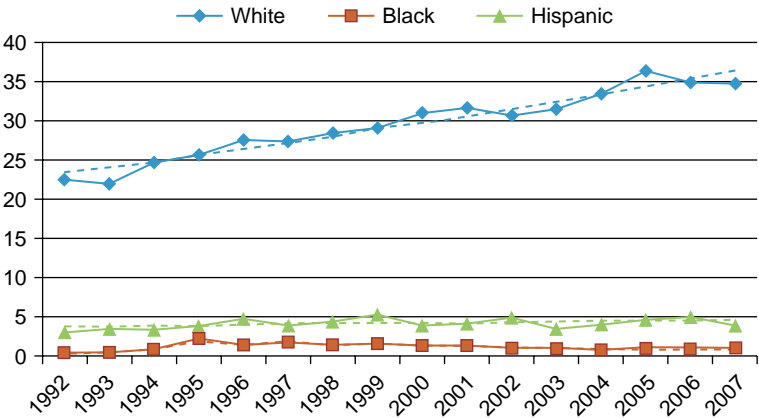


Fig. 3.1 Age-adjusted melanoma incidence rates* per 100,000 for males, by race/ethnicity. “White” refers to “non-Hispanic white” and “Hispanic” refers to “white Hispanic” (*Standardized to the US 2000 population)

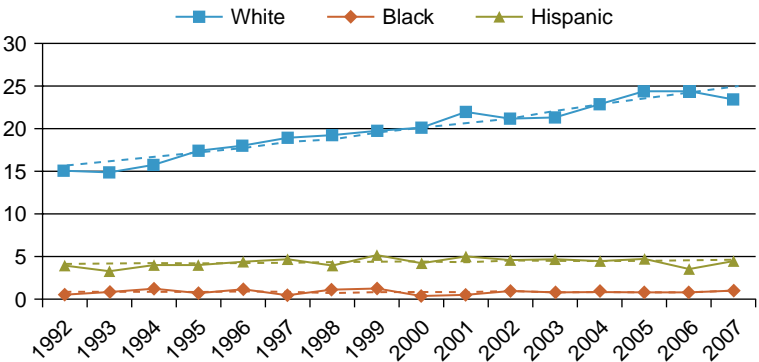


Fig. 3.2 Age-adjusted melanoma incidence rates* per 100,000 for females, by race/ethnicity. “White” refers to “non-Hispanic white” and “Hispanic” refers to “white Hispanic” (*Standardized to the US 2000 population)

both groups represent a large proportion of the NCI SEER (Surveillance, Epidemiology, and End Results) analytic group, it can be seen that overall the age-adjusted melanoma incidence rates do not reflect an increase overall among those who are identified as Hispanic, in either males or females.

When melanoma is identified among racial/ethnic groups other than Whites, it is often at a deeper Breslow thickness and more advanced stage. This may be due to a lack of awareness of melanoma. Unfortunately, but logically, most risk models (i.e., Fears et al. 2006) and melanoma awareness campaigns have been developed for white subjects, those at highest risk.

Melanoma mortality has continued to increase among white males worldwide. Over three decades (1969–1999), mortality rates from melanoma increased 157% in men aged 65 and older (Geller et al. 2002) (Fig. 3.3). However, there is a trend toward a plateau among females as noted in both the Australian data and the US data (Fig. 3.4). Unfortunately, this trend is not evident worldwide. In the UK, for example, the increase in mortality among women is continuing although it too is slowing.

A thoughtful analysis by Erickson and Driscoll (2010) suggests that the discrepancy between a rapid increase in incidence and slower increase in mortality in combination with the increase in thick minimally invasive melanomas relative to thicker melanomas may be the result of intensive surveillance and a concomitantly increased biopsy rate, or “overdiagnosis” (suggested by Welch and Black 2010). Overdiagnosis can be noted when the curve for incidence is relatively steep and that for mortality is flat or relatively so. This situation exists in Australia and the United States, although mortality is not actually flat, but is continuing to increase among the older age-groups.

Recent analyses indicate that although the distribution of tumors by thickness has remained relatively stable, those thick tumors, likely to result in death, have actually increased (Criscione and Weinstock 2010). In addition, there has been an approximately doubling of in situ tumors between 1988 and 2006. Until we are able to distinguish the

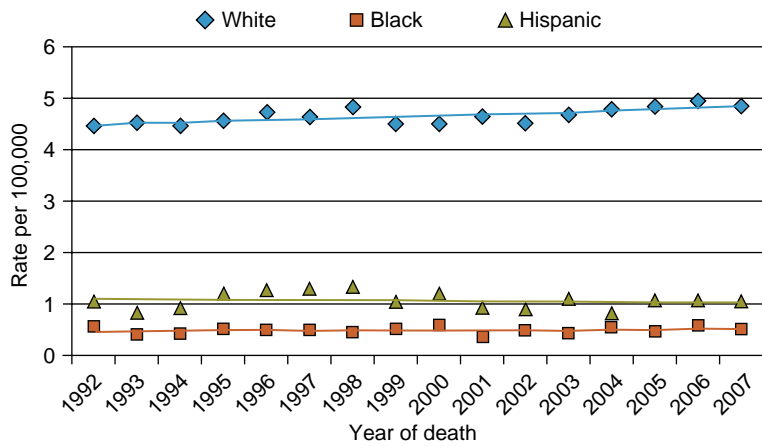


Fig. 3.3 Age-adjusted melanoma mortality rates* per 100,000 for males, by race/ethnicity. “White” refers to “non-Hispanic white” and “Hispanic” refers to “white Hispanic” (*Standardized to the US 2000 population)

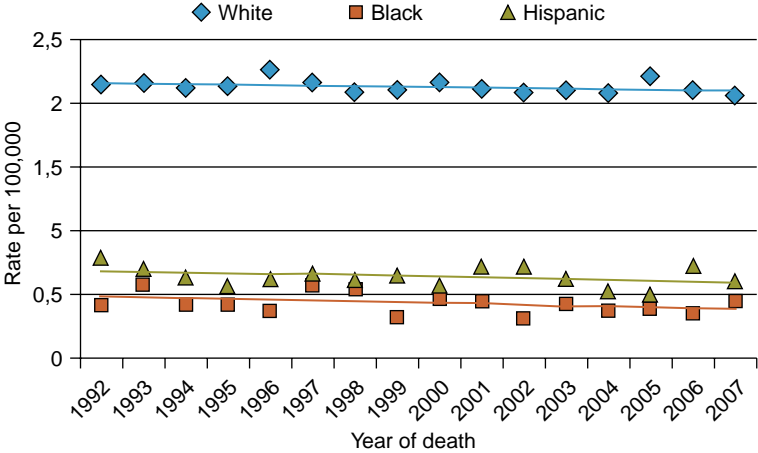


Fig. 3.4 Age-adjusted melanoma mortality rates* per 100,000 for females, by race/ethnicity. “White” refers to “non-Hispanic white” and “Hispanic” refers to “white Hispanic” (*Standardized to the US 2000 population)

faster growing, more aggressive tumors that are likely to lead to death from the more slowly growing tumor that may never cause problems, we will not be able to address this issue in a way to improve public health. Several investigators (Liu et al. 2006; Grob et al. 2002) have made attempts at evaluating these aggressive from more slowly growing tumors; however, these methods are not yet standard and are unlikely to be applied globally.

3.3 Host Factors

3.3.1 Age and Sex

Lachiewicz and colleagues (2008) describe melanoma as a heterogeneous cancer with tumors with different biological mechanisms having different survival patterns. In an analysis of age-specific incidence rates, it is clear that there are two peaks of incidence in melanoma, one at 54 years and one at 74 years, with truncal melanoma peaking nearer the first age and melanoma of the face and ears peaking in the later age period. The median age of melanoma diagnosis ranges between 57 and 62 years. Unfortunately, melanoma among young women has been recently increasing in the US and is currently the cancer with the highest incidence among young women aged 15–24 (Purdue et al. 2008), and the site with the highest incidence is the trunk (Bradford et al. 2010). In the US the age-specific incidence of melanoma among young women has generally been higher than that of males under the age of 40.

This pattern of the age-specific melanoma incidence is quite distinct for males and females, with a higher incidence among females up to the age of 45–50 when the age-specific rate for males climbs steeply while that for females continues to increase at a far slower rate.

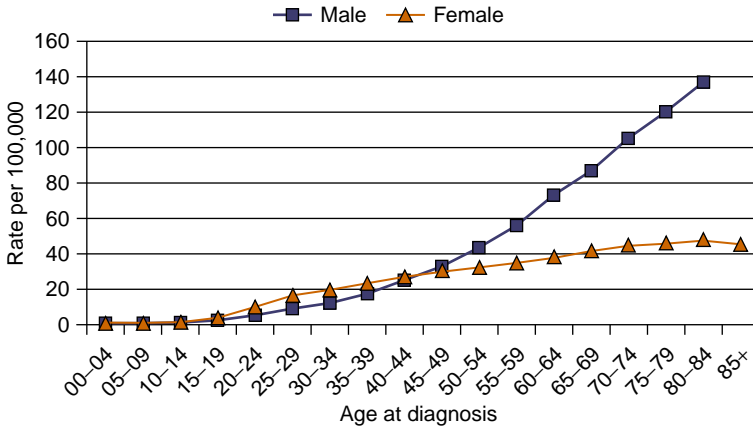


Fig. 3.5 Average annual age-specific incidence rates per 100,000. Non-Hispanic white residents of 13 core SEER program areas, 1992–2007

A “Clemmensen’s hook” has been described for melanoma incidence, a similar pattern as seen in breast cancer, where there is a change in the slope of incidence near menopause, indicating a potential role for female hormones in the etiology of the disease (Fig. 3.5). It should be pointed out that while Australia’s melanoma incidence rates are approximately three times those in the US, they are approximately nine times those in Ireland (Table 3.1). This statistic is rather startling and underlines the critical importance of the mixture of phenotype and intense solar exposure in Australia where much of the population migrated from Ireland and the UK. Given the great difference in incidence rates, the similarity of the age-specific rates between the US and Australia is all the more striking.

3.3.2

Melanoma and Pregnancy

Given the survival advantage of females and the presence of a Clemmensen’s hook in the incidence rates as well as the consistent pattern of age-specific incidence rates between an area of high incidence (Australia) and moderate incidence (the US), much speculation has focused on hormonal aspects of melanoma. The role of pregnancy and melanoma incidence and survival has received the most attention. The bulk of evidence amassed over the past half century, however, suggests that pregnancy does not significantly affect the risk of developing malignant melanoma. Further, pregnancy does not appear to adversely influence overall survival from the disease. Results from some studies suggested that pregnant women with melanoma were more likely than their nonpregnant counterparts to exhibit adverse prognostic indicators, specifically, thicker lesions and shorter time to recurrence. Nonetheless, most studies found no difference in overall survival between pregnant and nonpregnant women with melanoma (Wiggins et al. 2005). Recent reports from large-scale, population-based studies support these conclusions. Newly discovered estrogen receptors have led to new hypotheses about the role of estrogen in melanoma and these are under intense investigation (Di Giorgi et al. 2009).

Table 3.1 Rates for age-standardized^a melanoma incidence (per 100,000), by sex, in countries selected for high-quality data and more than 100 cases per year. (IARC, 2007, Cancer Incidence in Five Continents, Volume IX)

Country	Males	Females
<i>America: Central and South</i>		
Brazil, Sao Paulo	6.5	5.7
Colombia, Cali	3.5	2.9
<i>America: North</i>		
Canada, excluding Quebec, Yukon, and Nunavut	10.9	9.3
USA, SEER (14 Registries)	14.8	10.5
USA, SEER (14 Registries): Asian and Pacific Islander	1.0	0.9
USA, SEER (14 Registries): Black	0.9	0.6
USA, SEER (14 Registries): Hispanic White	3.0	3.2
USA, SEER (14 Registries): White	19.4	14.4
<i>Asia</i>		
China, Hong Kong	0.7	0.6
India, Bombay	0.3	0.2
Israel: Jewish	12.2	10.5
Japan, Osaka Prefecture	0.4	0.2
Turkey, Izmir	1.3	1.0
<i>Europe</i>		
Austria	8.6	7.8
Belarus ^b	2.7	3.5
Belgium, Flanders ^b	5.5	8.8
Croatia	6.9	5.8
Czech Republic	9.9	8.8
Denmark	11.9	14.1
Estonia	5.3	6.6
Finland	8.8	7.5
France, Bas-Rhin ^b	11.3	12.1
Germany, Munich	12.7	11.5
Iceland	9.3	19.0
Ireland	7.4	11.0
Italy, Torino	10.7	11.4

Table 3.1 (continued)

Country	Males	Females
Latvia	3.2	4.2
Lithuania	3.7	5.2
Norway	14.2	14.6
Poland, Cracow	5.7	5.6
Portugal, South Regional	3.7	4.8
Slovenia	9.0	8.7
Spain, Tarragona	6.4	8.7
Sweden ^b	11.9	12.1
Switzerland, Vaud	16.6	19.6
The Netherlands	10.0	12.9
UK, England, Oxford Region	9.8	12.6
UK, Scotland	8.4	10.1
<i>Oceania</i>		
Australia, Queensland	55.8	41.1
New Zealand	34.8	31.4
USA, Hawaii	13.6	8.3
USA, Hawaii: White	41.1	26.3

^aStandardized to the world population, 1960

^bPercentage reporting by death certificate only was not provided by the source dataset

3.3.3

Body Site

In addition to the distinctly different pattern of incidence between males and females, the anatomic site for the development of melanoma varies distinctively among males and females. Many observers feel that this is due to specific patterns of sun exposure, but a minority think that there may be a sex-linked genetic factor that influences the distribution of melanomas by anatomic site. This conjecture is yet to be proven. In almost every registry in the world, women have a preponderance of melanomas on the leg while males have a majority of melanomas on the trunk. This difference has been modifying over time as women develop more melanomas on the trunk. Lachiewicz et al. (2008) demonstrated, as others have previously, that males and females have different incidences of melanoma on the trunk and head and neck, with the male excess on the trunk occurring at approximately age 54, while the head and neck incidence among males peaks at age 77.

These data are consistent with Whiteman's "divergent pathway" model (Whiteman et al. 2003) where those with an inherently low propensity for melanocyte proliferation require chronic sun exposure to habitually exposed sites, such as the face, to develop melanoma, whereas those with a high propensity for melanocyte proliferation develop melanomas on sites with unstable melanocytes – or aberrant melanogenesis, such as the trunk – with intermittent solar damage.

3.3.4

Pigmentation

As can be noted in Table 3.1, melanoma occurs most often among light-skinned individuals. Melanin type and content of both melanocytes and keratinocytes is critical for determining skin phenotype. Individual photoprotection of the skin is based primarily on the level of constitutive, or genetically determined, pigmentation afforded by the types and amounts of melanin synthesized and distributed in the skin. Constitutive pigmentation of the skin with higher melanin content protects the epidermis from DNA damage (Yamaguchi et al. 2006). The melanocortin 1 receptor (*MC1R*) seems to regulate the activity of melanocytes and thus is a critical genetic factor in melanin synthesis and is discussed in Chap. 2.

Those with light hair, light eyes, and skin that burns easily are at most risk for developing melanoma from UV exposure whether it is from the sun or from artificial tanning devices. To the extent that young women with these characteristics feel they are "safe" using artificial tanning devices, they need to be warned of the dangers of overexposure to all UV. Most studies reviewed did control for phenotypic factors and some for recreational sun exposure, but even then, this procedure is unlikely to achieve "complete control."

Genetics plays a role in risk to any UV and all the factors involved have not yet been determined. Recent publications show that those exposed to solar UV have a wide variety of responses to UV (see Tran et al. 2008) in terms of cellular response to DNA damage, DNA repair capacity (Wei et al. 2003), and vitamin D synthesis due to Vitamin D Receptor polymorphisms (Santonocito et al. 2007), among others.

It is critical to note that individuals with similar levels of constitutive pigmentation may have different responses to UV (Bykov et al. 2000; Wagner et al. 2002). These differences may be due to variation in pigmentation genes. Data from the University of Pennsylvania (Kanetsky et al. 2010) and Australia (Palmer et al. 2000) demonstrate that pigmentary phenotype alone is not an adequate indicator of melanoma risk. Individuals who display darker phenotype characteristics (dark hair, brown eyes, and ability to tan) and who carry any variant *MC1R* alleles show an increased risk for melanoma.

3.3.5

Nevi

An important pigmentary-related factor is nevus density; individuals with many nevi are at consistently higher risk for developing melanoma. In fact, a large number of nevi have been shown to be the strongest known risk factor for developing melanoma among

Caucasians (Armstrong and Kricger 2001). Even in the absence of clinically atypical nevi, a very high number of nevi (e.g., more than 100) have been shown to significantly increase risk for melanoma (Huynh et al. 2003). Patients with great many nevi may be missing a genetic checkpoint. The absence of this checkpoint may permit the development of a higher number of nevi and increase melanoma risk. Nevus density is a simple characteristic that is likely to be useful in determining risk for melanoma among all persons and measuring genetic factors underlying nevus density should improve risk estimation.

There is an apparent interaction between sun exposure and nevus density with regard to the site of the melanoma. For example, in Australia, sex differences in nevus density on the back and lower extremities are similar to sex differences for melanoma – men having higher rates on the back, women having higher rates on the legs – areas that are not chronically exposed to the sun (Green 1992). Whiteman et al. (2003) have proposed a model for cutaneous melanoma in which two pathways – chronic exposure to the sun and melanocyte instability – represent divergent pathways for developing melanoma. Under this model, people with an inherently low propensity for melanocyte proliferation require chronic sun exposure to drive clonal expansion of transformed epidermal melanocytes. Melanomas arising in this group of people would occur on habitually sun-exposed body sites, such as the face. In contrast, the model would predict that in individuals with an inherently high propensity for melanocyte proliferation (e.g., high nevus counts), exposure to sunlight early in life would be required to start the process of carcinogenesis. These individuals would be expected to develop tumors on body sites with unstable melanocyte populations such as the trunk.

3.3.6

Family History

First-degree relatives of melanoma patients have a higher risk of the disease than individuals without positive family history (Greene and Fraumeni 1979), suggesting that a distinct hereditary component exists. Familial melanoma accounts for an estimated 5–10% of all cases of melanoma, and characteristics which distinguish the familial from the nonfamilial form of the disease include younger age at first diagnosis, better survival, thinner lesions, multiple primary lesions, and increased occurrence of non-melanoma cancers (Kopf et al. 1986). Ford pooled data from eight case–control studies and found that an individual's risk of melanoma increases by about twofold if he has an affected first-degree relative (Ford et al. 1995), and this effect was independent of host factors such as age, nevus count, hair and eye color, and freckling. Familial relative risk remained similar in all of the studies, even though melanoma incidence varied by about 10-fold in the study areas.

3.3.7

Immunologic Factors

A major enigma is that the host immune system is clearly associated with the development and progression of melanoma, but the mechanism by which it does so is not established. In some studies, melanoma incidence increased in frequency and aggressiveness after organ

transplantation and immunosuppressive therapy (Vajdic et al. 2009; Dinh and Chong 2007). There is also evidence that immune reactions are altered after UV exposure in the skin (locally) and perhaps throughout the body (systemically) (Murphy et al. 1993; Hersey et al. 1983; Norval 2006). Thus, melanoma represents a unique model for studying the human immune system, and the role of vitamin D in coordinating important changes in cancer development.

Some data are available as to how the human immune system may alter during or as a result of UVB radiation exposure (Bechetoille et al. 2007; Muller et al. 2008; Seité et al. 2003; Berthier-Vergnes et al. 2001). However, very little is known about how and why the immune surveillance mechanism actually fails to destroy melanoma precursor lesions. Melanomas are extremely antigenic, because melanoma cells produce high amounts of melanoma-specific proteins (Gould Rothberg and Rimm 2010). Even though certain T cell clones can recognize those proteins, they remain in a suppressed status; therefore, they cannot actively eradicate cancerous melanocyte growths. Lymphocytic infiltrates, mostly CD8+ or CD4+ CD25+ Fox3+ T regulator cells have been detected in and around tumor sites in several cancers (Nedergaard et al. 2007). Cytokines are also produced by these lymphocytes that are locally present in many types of cancers. However, there is little information available on the activity of these cells and their cytokine production in melanoma. It is generally thought that UV exposure can induce direct immunosuppression; however, there is no information on systemic and local immune reactions associated with the etiology of melanoma.

3.4

Environmental Factors

3.4.1

Sun Exposure

Sun exposure is generally equated with ultraviolet radiation exposure, although the evidence does not rule out other unmeasured exposures associated with the sun. In the public mind, a major correlation exists between increased outdoor activity and increased skin cancer rates. In fact, there are no data available to substantiate such a relationship; that is, although there has been a dramatic increase in melanoma incidence over the last 50 years, no data show that has been an increase in outdoor activity during the past 50 or so years although the trend toward wearing less clothing is self evident.

The data to support an association between sun exposure and the development of melanoma are indirect. There has been a latitude gradient for the incidence of melanoma among Caucasians, such that the highest rates are nearest the equator. In Europe this gradient has been confounded by the fact that those with darker pigmentary phenotype live in the Southern areas of Europe and those with lighter phenotype in the Northern, so that the gradient in Italy, for example, was actually reversed. However, this does not explain the higher melanoma rates in Norway than in Sweden. Furthermore, new data suggest that trends for incidence and mortality are “evening out” in terms of latitude. Armstrong and Kricker (1993) estimate that between 68% and 90% of all melanomas are caused by sun exposure. Most would not dispute this estimate; however, the major point here is that it is likely

intermittent sun exposure among susceptible individuals that leads to melanoma as noted among the UK migrants to Australia.

3.4.1.1

Patterns of Sun Exposure: Intermittent, Chronic, and Cumulative Sun Exposure

Although there is no standard measure of sun exposure in research, sun exposure can be generally classified as “Intermittent” or “Chronic,” and the effects may be considered as Acute or Cumulative. *Intermittent sun exposure* is that obtained sporadically, during recreational activities usually, and particularly by indoor workers who have only weekends or vacations to be outdoors and have not adapted to the sun. *Chronic sun exposure* is incurred by consistent sun exposure, usually by outdoor work, but also among those people who are outdoors a great deal. *Cumulative sun exposure* is the additive amount of sun exposure that one receives over a lifetime. Cumulative sun exposure may reflect the additive effects of intermittent sun exposure or chronic sun exposure or both. Thomas et al. (2010) recently demonstrated that solar elastosis, a breakdown of collagen and elastin in the epidermis, when located near the site of a melanoma is directly linked to high levels of ultraviolet radiation at the site. Surprisingly, Berwick et al. (2005) demonstrated that solar elastosis is associated with better survival from melanoma, independent of age or histologic subtype of the tumor.

Indeed, different patterns of sun exposure appear to lead to different types of skin cancer among susceptible individuals. In Europe Rosso et al. (1996) quantified suggestions by Krickler et al. (1995) that basal cell carcinoma and squamous cell carcinoma have different patterns such that squamous cell carcinoma appears to have a threshold at approximately 70,000 h of exposure to sun after which incidence increases sharply, regardless of whether it is chronic sun exposure or intermittent sun exposure. This is highly consistent with the molecular genetic evidence (Kraemer et al. 1994) where combined analysis of skin cancer mutations from several laboratories found the p53 tumor suppressor gene mutated in 90% of human squamous cell carcinomas and approximately 50% of basal cell carcinoma. Approximately 70% of tumors exhibited the characteristic UVB footprint, a C to T or a CC to TT mutation at specific codons.

It is a surprise to many that analytic epidemiologic studies have shown only modest risks at best for the role of sun exposure in the development of melanoma incidence, and three systematic reviews have demonstrated extremely similar estimates of effect for the role of intermittent sun exposure, an odds ratio of 1.6 (Gandini et al. 2005; Nelemans et al. 1995; Elwood and Jopson 1997). It is important to note that chronic sun exposure, as in those occupationally exposed to sunlight, is protective for the development of melanoma, with an odds ratio of 0.7–0.9, equivocal for the development of basal cell carcinoma, and a risk factor for squamous cell carcinoma. As Elwood and Jopson point out, the measurement of sun exposure is complex and any discrepancies among studies could be sorted out by conducting new studies using compatible protocols in different populations with different levels of sun exposure.

A clearer explanation for the rise in melanoma incidence that takes into account the different effects of chronic and intermittent sun exposure, proposed by Gallagher et al. (1989), is that as people have replaced outdoor occupations with indoor, they have engaged in more intermittent sun exposure. Gallagher showed that the decrease in

outdoor occupations, or chronic exposure which is not a risk factor for melanoma, could explain the increase in melanoma incidence in Canada.

The evidence for cumulative exposure comes from two sources to date: migrant studies and studies of lifetime exposure, controlling for intermittent and occupational exposure. Data from Australia and Italy show that individuals who migrate at a young age (less than 10 years) from areas of low exposure, such as the UK, to areas of high exposure, such as Australia or Israel, have a lifetime risk of developing melanoma that is similar to that of the new country. On the other hand, individuals who migrate later in life, adolescence or older, from areas of low solar exposure to areas of high solar exposure, have a risk that is quite reduced. These data have often been cited to indicate that childhood sun exposure is more important than adult sun exposure in the development of melanoma. However, they can also be interpreted to indicate that the length of exposure is critical rather than the time of exposure; that is, those who migrate early in life have a longer period for intense exposure compared to those who migrate later in life.

3.4.1.2

Effect Varies by Skin Type

The pattern of sun exposure that appears to induce melanoma development is complex and is clearly different by skin type (i.e., propensity to burn, ability to tan). Armstrong et al. (1997) have proposed a model consistent with data from other epidemiologic studies (White et al. 1994, among others) where risk for melanoma increases with increasing sun exposure among those who tan easily, but only with a small amount after which risk decreases with increasing exposure. Among subjects who are intermediate in their ability to tan, risk continues to increase slowly and then at some point declines with increasing exposure. On the other hand, those subjects who have great difficulty tanning have an almost linear increase in risk with increasing sun exposure. This model recognizes that individuals are differentially susceptible to sun exposure and have different levels of risk based on skin type. Moreover, it suggests that different types or patterns of sun exposure are associated with different levels of risk for melanoma.

It is worthwhile looking at the estimates of effect of sun exposure on the development of melanoma in tandem with the other major risk factors for the development of melanoma – nevi number and pigmentary phenotype. Work is ongoing to determine the interrelationship of genetic susceptibility and these phenotypic characteristics (Begg and Berwick 1997). In unpublished data from a population-based case control study in Connecticut (Berwick et al. 1996), the investigators estimated the risk for developing melanoma for nevus number, pigmentary phenotype, and sun exposure in early life as well as sun exposure 10 years prior to the diagnosis of melanoma, adjusting for age and sex. The risk for melanoma with numerous nevi in this study is six times that of someone with few nevi. The risk for melanoma with the most sensitive pigmentary phenotype is almost six times that of someone with the least sensitive phenotype. However, the risk for melanoma with increasing early life sun exposure or increasing later life sun exposure is only two times that of someone with the least sun exposure. Clearly, genetically determined characteristics such as nevi and pigmentary phenotype are more powerful determinants of melanoma risk than is sun exposure.

3.4.1.3

Sunburn

The role of sunburns in the development of melanoma is a critical issue. This aspect of sun exposure is the one most often cited as key to determining melanoma risk. In fact, it can be forcefully argued that sunburn itself is not on the pathway to the development of melanoma, but that it is an important marker for the combination of genetically susceptible phenotype and excessive sun exposure. Numerous articles in the lay media as well as dermatology journals stress the importance of a specific number of sunburns in increasing risk for melanoma. However, a critical look at these studies will show that the relative risk for developing melanoma, when adjusted for host characteristics, is often not statistically significant and is not always impressive. Recent data from Europe (Autier and Dore 1998) support the concept that childhood sun exposure is not well represented by sunburning episodes.

While sunburn is the most visible and immediate effect of overexposure to UV, it is also the one that the public is most likely to associate with the development of melanoma. The emerging consensus, however, is that it is unlikely that sunburn is causally associated with melanoma; it is more likely that sunburn is a clear indicator of the interaction between too much sun exposure and a susceptible phenotype, that is severe solar exposure to skin unaccustomed to it.

3.4.1.4

Suberythemic Exposure

Indeed, a great deal of research is currently being focused on suberythemic exposures, that is those doses of ultraviolet radiation that do not cause an actual burn, but that may have biologic significance. Certainly, exposures to the UVA portion of the UV spectrum may lead to the development of melanoma (Moan et al. 1999).

Measurement error is a more serious problem in evaluating sunburn history than other sun-associated variables (English et al. 1998; Westerdahl et al. 1996; Berwick and Chen 1995). At least three studies have conducted test–retest reliability studies and concluded that sunburn history is poorly recalled with only a little over half the subjects giving the same answer at two points in time to the question: “Have you ever been sunburned severely enough to cause pain or blisters for 2 days or more?” Other sun-associated variables, such as time spent outdoors during recreation, for example, appear to be more reliably remembered (English et al. 1998).

3.4.1.5

Timing of Sun Exposure

Much has been made of the critical time of sun exposure in the development of melanoma. This concept has not yet been proven. In fact, it is highly likely that all stages of development are important in the development of melanoma. Data from a case–control study of melanoma conducted in Connecticut show that intermittent exposure in the 10 years prior

to the diagnosis of melanoma is just as important as intermittent exposure in early life. The argument that 70% of one's sun exposure is likely obtained before the age of 20 may be true; however, this often-quoted statistic is merely an estimate. With the changes in lifestyle of the 1990s and the early twenty-first century, it is quite possible that individuals in the latter half of life receive a very substantial amount of sun exposure as a result of early retirement and flexible work schedules. At the same time, there are numerous forces at work to diminish the outdoor experiences of young people: the tremendous increase in video games and computers as well as the increasing atomization of neighborhoods, so that "pick up" games of kick the can are no longer as easy to organize.

The preponderance of data show that excessive intermittent sun exposure at any age increases risk for melanoma. Although the public and many researchers feel that sun exposure during early childhood is the critical period for melanoma induction, there are not empirical data to support this view. It surely is an attractive view.

Autier and Dore (1998) attempted to address the issue as to whether early life or later life sun exposure was the critical factor in determining melanoma risk. They found that both time periods were important. An interesting comparison shows the joint effects of sun exposure during childhood and adulthood. They find, as one might expect, that the highest risk among adults is for those who had high intermittent sun exposure as children. Conversely those who had low sun exposure during childhood and high sun exposure in adulthood had a similar risk to those who had high exposure during childhood and low exposure during adulthood.

Other data support the idea that intermittent sun exposure leads to increased risk at any age. Holly et al. (1995) showed that more than seven painful sunburns during elementary school increased risk twofold ($OR=2.0$, 95% $CI=1.4, 2.9$) and that more than seven sunburns after the age of 30 (the age of women in this study ranged from 18 to 59) increased risk twofold ($OR=2.0$, 95% $CI=1.1, 3.8$). In sum, data from very different setting seems to suggest that intermittent sun exposure is critical to the risk for developing melanoma. In the published studies that looked at both early life and adult sun exposure, there is very little difference between the effects of sun exposure at either stage, but that lifelong intermittent sun exposure is indeed cumulative.

3.4.1.6

Occupational or Chronic Sun Exposure

The reasons for the differing trends in melanoma risk between occupational and intermittent sun exposure are not well understood. Analyses of melanoma time trends from Canada (Bulliard and Cox 1999), New Zealand (Bulliard and Cox 2000); Germany (Garbe et al. 1994); Australia (Garbe et al. 2000; Marrett et al. 2001), and Denmark indicate that changes in lifestyle factors, such as sun exposure behaviors and fashion, correlate (Osterlind et al. 1988) strongly with increases in melanoma on skin areas exposed intermittently to the sun (trunk, upper arms, and upper legs). With regard to chronic occupational sun exposure, it is also possible that additional phenotypic differences among workforce members may be influencing the direction and intensity of melanoma risk. In a study of occupational melanoma from Spain (Espinosa Arranz et al. 1999) higher melanoma risk was observed among construction workers than among farmers. The melanoma risk in construction workers

became more significant when adjusted for skin type, age, freckle count (Odds Ratio (OR) 4.3; 95% Confidence Interval (CI) 1.8, 9.9), and number of nevi (OR 2.8; 95% CI 1.4, 5.8), while the risk in farmers remained protective even with these adjustments.

3.4.2

Tanning Lamps

Tanning lamps have been increasing in number and popularity throughout the world, particularly but not exclusively in more northern latitude. Great concern has been expressed by the International Agency on Cancer (IARC) that this increase will lead to increases in melanoma risk. IARC convened an expert panel of epidemiologists (IARC 2007) who performed a meta-analysis of 19 studies that have evaluated the association between sunbed exposure and melanoma and other skin cancers to that time. This analysis showed a significant summary, or overall, relative risk for melanoma of 1.8 (95% CI 1.4, 2.3) for “first exposure under the age of 35”; a relative risk of 1.2 that was statistically significant (95% CI 1.0, 1.3) for “ever use.” Controversy has, however, continued over the carcinogenic properties of tanning beds. The tanning industry “sells” tanning beds as a safe alternative to UV exposure for both tanning as well as vitamin D biosynthesis.

A recent study conducted in Minnesota has published very high and significant risks for developing melanoma among tanning bed users. Among 1,167 cases and 1,101 controls, 62.9% of cases and 51.1% of controls had tanned indoors (adjusted OR 1.7; 95% CI, 1.4, 2.1). Melanoma risk was pronounced among users of both UVB-enhanced (adjusted OR, 2.9; 95% CI, 2.0, 4.0) and primarily UVA-emitting devices (adjusted OR 4.4; 95% CI, 2.5, 8.0). Risk increased with use: years ($P < 0.006$), hours ($P < 0.0001$), or sessions ($P = 0.0002$). ORs were elevated within each initiation age category; among indoor tanners, years used was more relevant for melanoma development (Lazovich et al. 2010). Thus, epidemiologic data suggest that tanning beds are not safer than solar ultraviolet radiation and that they may have independent effects from solar exposure that increase risk for melanoma.

A major problem in evaluating the risk from tanning beds and sunlamps is that they have changed over time in terms of their usage and their spectral output. In addition, the dosage of UV is extremely difficult to obtain as most tanning parlors do not calibrate their equipment or measure their output. When comparing dosage of tanning lamps to solar radiation, it is important to estimate the proportion of the body irradiated. From 15% to 50% of the total body is uncovered during outdoor activities, but up to 95–100% of the total body is uncovered during indoor tanning. Therefore, the dosage is likely to be far greater than from a similar amount of outdoor solar exposure.

Females tend to use sunbeds more than males, particularly young women. Recent data from the United States National Cancer Institute show that the incidence of melanoma is growing among young females (Purdue et al. 2008). In addition, sunbed usage in the US is most prevalent among young women (Lazovich and Forster 2005). These statistics point up the fact that sunbed usage is an area for serious concern. In fact, Veierød et al.’s (2003) evaluation of use of sunbeds found that those who used sunbeds at ages 20–29 years once or more per month had a statistically significant relative risk of developing melanoma of 2.6 (95% CI 1.5, 4.5). This fact will be crucial to guide prevention in the future.

Most studies have shown an increased risk for melanoma associated with sunbed use, but there are multiple qualifications that need to be taken into account. In the first place, it is difficult to disentangle the use of artificial UV from natural UV exposure. Many authors, for example, Wester et al. (1999), have found frequent tanning in sunlight correlated with sunbed use.

Gallagher et al. (2005) asks the critical question: “If there is a causal relationship, how important is the risk?” This is a difficult question to respond to at this point in time for a number of reasons: (1) Assessment of sunbed use needs improvement as well as assessment of spectral output. Although we see good agreement for individual’s recall of sunbed usage, it is likely that the timing and the exposure are not all the same for all individuals. (2) It is unclear whether one can compare sunbed use and sunbathing; is there the same biological mechanism? (3) Most studies have taken place in higher latitudes in North America and Europe where the background ambient ultraviolet radiation is low; it would be useful to have more data from lower latitudes with higher levels of ambient UV, such as Australia and Southern US. (4) Ecological studies are inconsistent – even at similar latitudes with very good data. In Denmark Faurschou and Wulf (2007) concluded that sunbed risk for BCC is important, but not CMM. However, in the UK, Diffey (2007) estimated that sunbed risk for CMM in women is dramatic and may have caused as many as 182 cases of CMM in women and 49 in males in the last 8 years.

3.4.3

Other Environmental Factors

Other environmental factors are receiving more attention with ongoing stronger study design.

3.4.3.1

Pesticides

Dennis’ group evaluated data from the Agricultural Health Workers’ Study and found significant associations between cutaneous melanoma and maneb/mancozeb (trend $P=0.006$), parathion (trend $P=0.003$), and carbaryl (trend $P=0.013$). Other associations with benomyl and ever use of arsenical pesticides were also suggested (Dennis et al. 2010).

3.4.3.2

Arsenic

Arsenic has been suspected of involvement, since it is a known cocarcinogen with UV in exacerbating development of non-melanoma skin cancer (Rossman et al. 2004). However, most elevated levels of As are found in areas with populations who are highly resistant to UV-induced melanoma (Taiwan, Bangladesh) and so even a significant increase in malignant melanoma skin cancer rate would not be detected in epidemiology studies in those

populations. A significant positive association has been shown between body arsenic levels (toenail arsenic) and melanoma risk in a (predominantly) Caucasian Iowa population, demonstrating that interactions between sunlight and arsenic may contribute to melanoma in these populations (Beane Freeman et al. 2004).

3.4.3.3

Polychlorinated Biphenyls (PCBs)

Some epidemiologic studies have suggested that exposure to organochlorine compounds might increase the risk of melanoma, but these studies have lacked biological measures of exposure and have not been able to control for the major environmental risk factor for melanoma – sun exposure. Gallagher and coworkers (2010) were able to conduct a pilot study with the ability to adjust for sun sensitivity and sun exposure. Plasma was collected from 80 cases and 310 controls in British Columbia. Assays for 14 PCB congeners and 11 organochlorine pesticide residues were conducted: risk of melanoma and plasma levels of non-dioxin-like PCBs (Adjusted OR 7.0, 95% CI 2.3, 21.4 among those in the highest quartile) as well as several PCB congeners, organochlorine pesticides, or metabolites. This study suggests that other environmental factors in addition to UV radiation may play an important role in the etiology of melanoma.

3.5

Summary

Melanoma is clearly a complex disease for which we do not have simple answers. This chapter has attempted to delineate some of the challenges in understanding the trends for melanoma incidence and mortality. Although we do not yet understand fully the etiology of melanoma, there seems to be a relatively clear message for the public: “Be cautious all your life. Enjoy the sun in moderation. Stay away from large bursts of sun exposure, particularly on untanned skin.”

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Abstract Over the previous decade, our knowledge in somatic genetic events related to melanoma progression has exploded. It has become increasingly clear that multiple genetic events are required for melanoma to develop and that melanoma is a vastly heterogeneous disease. Signaling pathways that are essential for tumor progression have been pinpointed due to recent technological developments, which simultaneously identify attractive therapeutic targets in a disease characterized by consistent resistance to current chemotherapy regimens (see Chaps. 7 and 16). As has been implicated in many other malignancies, molecular profiling is anticipated to reveal prognostic, diagnostic, and predictive tools in the clinical setting of melanoma.

Additionally, tumor genetic data have recently been interconnected with epidemiology of melanoma (see Chap. 3). Two etiological pathways have been postulated to trigger the formation of melanoma. The first pathway involves relatively young individuals with large numbers of nevi and who develop melanomas on locations (e.g., trunk) that are intermittently UV exposed. This pathway is further corroborated by the fact that *BRAF* mutations, which frequently occur in nevi, are also more common in melanomas of younger patients with larger number of moles. A second pathway, which is probably related to chronic UV exposure, leads to melanomas that develop among older individuals and on chronically sun-exposed sites. Additionally, genetic analyses have uncovered a previously undisclosed hidden molecular structure between subtypes of melanoma.

Along translational lines, tumor genetic information has recently shown to be of enormous significance for personalizing therapeutic choice, e.g., Braf inhibitors that selectively target *Braf*^{V600E} mutated melanomas, thus tumor genetic information is required prior to treatment.

In this chapter, we have screened current literature for an update of genetics and genomics within the field of melanoma tumor biology.

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4.1

MAPK- and PI3K Pathway and Melanoma

There is substantial evidence that two of the major pathways central for melanoma development are the Mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinases (PI3K) pathways. The first oncogene to be identified in melanoma was *NRAS* (Padua et al. 1984), which is known to be mutated not only in melanoma but also in other cancers. It would take an additional 20 years before the next established melanoma oncogene was discovered. In a comprehensive mutation screen of protein kinases in human cancer conducted by the Wellcome Trust Sanger Institute, *BRAF*, the immediate downstream effector of *NRAS*, was found to be somatically mutated in >60% of metastatic melanomas (Davies et al. 2002). The dual role of *Nras* in activating both the MAPK- and PI3K-pathways and *Braf* in activating the MAPK-pathway underscores the importance and therapeutic opportunities the defects in these pathways presents. Here, we summarize the genetic alterations found in these pathways and their consequences.

4.1.1

MAPK-Pathway

Braf is a serine threonine kinase and a member of the “classical” MAPK-pathway, which regulates cell growth, survival, and differentiation. *Braf* is highly expressed in melanocytes and neuronal tissue, both of which are of neural crest origin. MAPK-pathway activation is mediated by receptor tyrosine kinases and G-protein coupled receptors, which subsequently activates the Ras family members and then the Raf kinase family (*Araf*, *Braf*, and *Craf*). The Raf kinases activate MEK1/2, which in turn stimulates Erk1/2 thereby triggering the eventual expression of genes involved in proliferation or differentiation (Fig. 4.1). It was demonstrated early on that mutations in *BRAF* was a common genetic event in nevus suggesting that it is an early event in melanoma progression (Pollock et al. 2003). This led several groups to investigate whether *BRAF* germline mutations were present in melanoma kindreds not accounted for by *CDKN2A* mutation; however, no germline mutations were identified thereby excluding the role of *BRAF* as a melanoma susceptibility gene (Casula et al. 2004; Jackson et al. 2005; Laud et al. 2003). The single most common mutation found in *BRAF* is the T1799A point mutation leading to an amino acid substitution of glutamic acid to valine at position 600 located in the kinase domain of the *BRAF* gene and in total >80% of all *BRAF* mutations occur at this locus (Hocker and Tsao 2007). Other putative oncogenic mutations occur at other codons in exon 15 or in exon 11, both of which correspond to the kinase domain as well (Hocker and Tsao 2007). The frequency of mutations in melanoma varies from 30% to 70% depending on the study; in the largest study on primary cutaneous melanomas, a *BRAF* mutation rate of 48.9% was reported (Ellerhorst et al. 2010). Noteworthy, *BRAF* mutation frequency differs in melanoma depending on certain clinical and pathological subsets. In melanomas arising from chronically sun-exposed body sites as

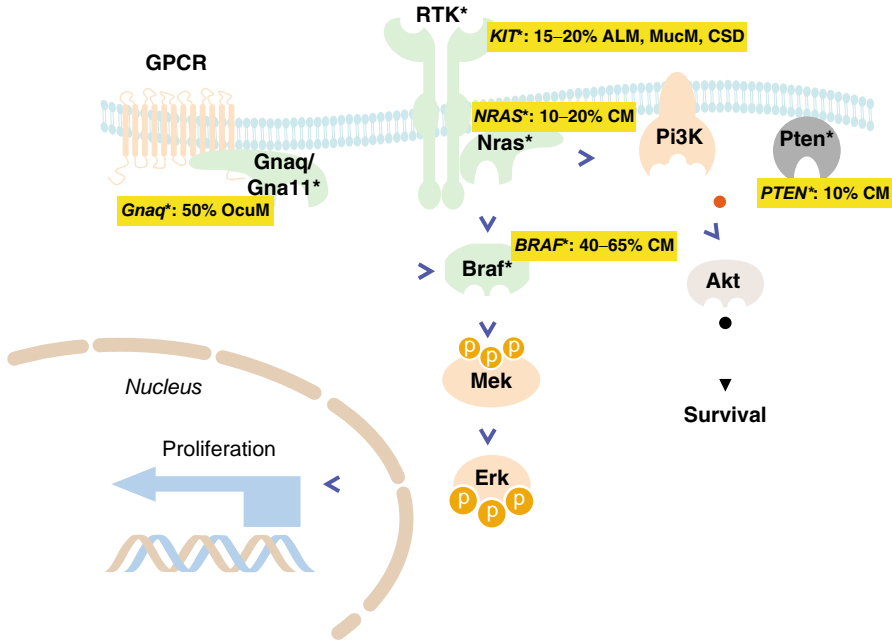


Fig. 4.1 Molecular Lesions in Melanoma Signaling Genes. Many components of the melanoma growth signaling cascade are mutated and activated. Genes are shown in *italics*, activating mutations are indicated by green shading, inactivating mutations are indicated by grey shading, and asterisks represent mutations. Percentages shown in yellow boxes summarize prevalence of mutations (RTK, receptor tyrosine kinase; GPCR, G protein-coupled receptor; ALM, acral lentiginous melanoma; MucM, mucosal melanoma; CSD, chronically sun-damaged melanomas; OcuM, ocular melanoma; CM, cutaneous melanoma)

well as in melanomas not related to sun exposure (mucosal and acral melanomas) the rate of *BRAF* mutagenesis is quite low, whereas the opposite is observed for melanomas arising on intermittently sun-exposed areas (such as trunk and back) (Curtin et al. 2005; Maldonado et al. 2003). Whether the difference is actually based on body site or histological subtype is difficult to tease apart since melanomas arising on chronically sun-exposed sites often are lentigo maligna melanomas (LMM) and melanomas on intermittently sun-exposed sites often are superficial spreading (SSM) or nodular melanomas (NMM) (Curtin et al. 2005). *NRAS*, the second most commonly mutated oncogene in cutaneous melanoma (~15%) is known to activate both the MAPK- and the PI3K-pathways (Ellerhorst et al. 2010). Most importantly, *BRAF* and *NRAS* mutations occur in a mutually exclusive manner and in all about 75% of all melanomas harbor mutations in either of the genes (Hocker and Tsao 2007). Additional RAS genes seem to have only a minor impact in melanoma. In recent studies, a few somatic mutations were detected in *KRAS* (Brose et al. 2002; Reifemberger et al. 2004). Likewise, *HRAS* mutations are rare in melanomas; however 12% of Spitz nevi harbor a genomic gain of the *HRAS* locus and simultaneously 67% of nevi harboring a gain

concomitantly carry a mutation in the *HRAS* gene (Bastian et al. 2000). In some rarer forms of melanoma, such as uveal melanoma, *BRAF* and *NRAS* mutations are extremely rare. Instead, mutations in the heterotrimeric *GNAQ* gene were identified in 46% of uveal melanomas. Mutations occurred at a single locus (*Gnaq*^{Q209L}) located in the Ras-like domain and *in vitro* studies verified that the Q209L mutation activated the MAPK-pathway suggesting an alternative route for *BRAF* and *NRAS* mutation (Onken et al. 2008; Van Raamsdonk et al. 2009). Moreover, *GNAQ* mutations are also frequently detected in certain type of nevi suggesting that it is an early genetic event similar to the findings of *BRAF* mutations in nevi (Van Raamsdonk et al. 2009). Additionally, *in vivo* analysis indicates that mutation of *GNAQ* is not sufficient for full progression to melanoma. This is also true for *BRAF* and *NRAS* mutations suggesting overlapping functional properties of these three proteins. The discovery of somatic mutations of *GNAQ* in uveal melanoma led research groups to investigate mutation patterns of heterotrimeric G proteins in melanoma. The group of Yardena Samuels examined the mutation spectrum of 35 genes in melanoma samples. Somatic changes were detected in 17% of all samples with *GNG10* and *GNAZ* having highest frequency of mutations (Cardenas-Navia et al. 2010). More recently, mutations in *GNAI1* were found in 32% of uveal melanomas in a mutually exclusive pattern with *GNAQ* mutations (Van Raamsdonk et al. 2010).

Genome-wide screens to discern whether molecular patterns associated to *BRAF* mutation exist have been performed. Pavay et al. used microarray expression profiling to distinguish *BRAF*- and *NRAS* mutant and wild-type cell lines. A set of 83 genes was identified to clearly discriminate *BRAF*-mutant and *BRAF* wild-type cells (Pavay et al. 2004), and the approach was later verified in additional datasets (Johansson et al. 2007). It has also been demonstrated that distinct genomic changes in *BRAF*-mutated cells where chromosomes 7 (harboring *BRAF*), 10q (harboring *PTEN*), 11q, 14q, and 20q were identified are discriminatory (Jonsson et al. 2007). The observation of concomitant *BRAF*^{V600} mutation and *PTEN* loss is further enforced by a significant association between *BRAF*^{V600} mutation and *PTEN* somatic mutation (Jonsson et al. 2007; Tsao et al. 2004). These genetic data are corroborated by a recent study where a mouse model with conditional melanocyte-specific expression of *BRAF*^{V600E} developed melanocytic hyperplasias. Moreover, when *PTEN* was abrogated mice developed melanomas with 100% penetrance and subsequently induced metastases in the lymph nodes and lungs (Dankort et al. 2009). These data emphasize the cooperativity between the PI3K- and MAPK-pathways for melanoma tumorigenesis and thus activating mutations of *BRAF* as well as inactivating changes in *PTEN* represent one molecular partnership to attain the desired effect. Taken together, these results suggest that *BRAF* mutant melanomas develop via a distinct genetic pathway.

Since the discovery of *BRAF* mutations, it has been speculated that this protein kinase can serve as an attractive therapeutic target. The first drug tested that targets Braf was Sorafenib, which was later found to be ineffective in the clinical setting (Eisen et al. 2006). More recently, Braf inhibitors such as PLX4032 – a drug which selectively targets Braf^{V600E} – mutated melanomas has shown promising results in phase I trials (Bollag et al. 2010; Flaherty et al. 2010) (see Chap. 16). Unexpectedly, *in vitro* studies have indicated that treatment of Braf wild-type cells with Braf inhibitors may in fact paradoxically enhance Erk signaling (Heidorn et al. 2010; Poulikakos et al. 2010) suggesting that genetic stratification of patients prior to treatment is absolutely essential.

4.1.2

PI3K-Pathway

As in the MAPK-pathway, *Nras* is also an essential part of the PI3K-pathway. Here, Ras induces membrane translocation and activates PI3K, which in turn leads to phosphorylation and activation of one of the major targets of the PI3K pathway, AKT. Mutations in members of the PI3K pathway have been extensively studied in several cancers. Although *PIK3CA* (p110 α subunit of PI3K) is a common oncogene in breast (~30%) and colon (~15%) cancers (Ogino et al. 2009; Saal et al. 2005), it is rarely altered in melanoma (Omholt et al. 2006). It appears that melanomas preferentially activate the PI3K pathway through inactivation of *PTEN*—another key component of the PI3K-pathway. *PTEN*, located on chromosome 10 is deleted in 30–50% of melanomas and is a major tumor suppressor gene commonly abrogated in human tumors. Somatic mutations of *PTEN* are rare in primary melanomas (Reifenberger et al. 2004; Tsao et al. 2004), whereas the frequency increases in metastases and melanoma cell line cultures (Guldberg et al. 1997; Pollock et al. 2002; Tsao et al. 2004); this may indicate a selective advantage for cells with a *PTEN* deficiency. There are three isoforms of AKT – AKT1, 2, and 3; however, there are several lines of evidence suggesting that Akt3 is the main player in melanoma. It has been shown that Akt3 is the predominant active form in melanoma cells and the *AKT3* gene is located on chromosome 1q a region commonly gained in primary melanoma. At least one metastatic tumor has been described to harbor focal amplification of the *AKT3* locus (Jonsson et al. 2007). Moreover, a rare point mutation (E17K) in the pleckstrin homology domain, which results in constitutive activation, was identified in melanoma cells (Davies et al. 2008).

Somatic mutations in one of the activators of the MAPK- and PI3K-pathways, *ERBB4*, were recently identified in a comprehensive screen of the tyrosine kinome (Prickett et al. 2009). ErbB4 is a tyrosine kinase receptor consisting of an extracellular domain, a α -helical transmembrane segment and an intracellular protein kinase domain. ErbB receptors require dimerization of two ErbB molecules, which leads to activation of the kinase domain that subsequently activates downstream targets. *ERBB4* was the most prevalently mutated gene in the tyrosine kinome screen; however *FLT1* and *PTK2B* were mutated in about 10% of investigated cases. Importantly, ErbB4 is an attractive therapeutic target in melanoma since a pan-ErbB small molecule inhibitor (lapatinib) is already available and is currently being evaluated in clinical trials in, e.g., breast cancer. Also, *in vitro*, melanoma cells harboring an *ERBB4* mutation were sensitive to lapatinib (Prickett et al. 2009).

4.1.3

The CDKN2A Network

The chromosome 9p21 locus is commonly targeted by loss-of-heterozygosity (LOH) or deletions in melanoma. The importance of this locus is underscored by the identification of somatic as well as germline mutations in *CDKN2A* (Hayward 2003; Jonsson et al. 2007). *CDKN2A* is unique in the way that it encodes for two different proteins, p16^{INK4A} and p14^{ARF}, each transcribed in a separate reading frame. The two genes share one exon but have two distinct first exons (exon 1 α and exon 1 β). The *CDKN2A* locus was discovered through linkage analysis of melanoma kindreds and molecular assays showing limited homozygous

deletions in some melanoma cell lines (Kamb et al. 1994; Weaver-Feldhaus et al. 1994). It was subsequently shown that germline mutations in *CDKN2A* could explain a substantial fraction of melanoma kindreds (Hayward 2003; Lin et al. 2008). In cultured melanoma cells *CDKN2A* is frequently inactivated by homozygous deletions whereas small deletions or mutations are more common as germline changes. For both germline and somatic mutations, many of the reported changes reside in exon 2, which impinges on both p16^{INK4A} and p14^{ARF} while mutations only affecting the p14^{ARF} transcript are rare. Within the retinoblastoma (RB) pathway, both the Rb protein and p16^{INK4A} act as tumor suppressors by regulating the cell cycle. Whereas Rb has been shown to have a limited role in melanoma development, p16^{INK4A} appears to be the major tumor suppressor in this pathway for melanoma. The key function of p16^{INK4A} is as an inhibitor at the G1-to-S cell cycle restriction point where it binds to Cdk4 and thus unable Cdk4 to bind to cyclinD1, which subsequently would lead to phosphorylation and inactivation of Rb. This ultimately releases the E2f transcription factors thereby transcriptionally upregulating S-phase-related genes. The p16^{INK4A} also plays an important role in cellular senescence, a mechanism that restricts the emergence of immortalized cells; however the explanation for this is elegantly reviewed elsewhere (Bennett 2003). In contrast, p14^{ARF} suppresses oncogenic transformation by binding and inhibiting Hdm2 whose function is to abrogate p53 function by targeting it for degradation via the ubiquitin pathway (Momand et al. 1992). Amplification of both *CDK4* and *MDM2* has been observed in a subset of melanomas and has also been associated with preserved expression of p16^{INK4A} and p14^{ARF} (Muthusamy et al. 2006). Additionally, melanomas with wild-type *BRAF* and *NRAS* frequently display increased gene copy number of *CDK4* and *CCND1*, which are direct downstream targets of the MAPK-pathway (Curtin et al. 2005). These melanomas frequently represent chronically sun-damaged, acral- or mucosal- type lesions. The relevance of the *TP53* gene is uncertain since 22% of melanoma cell lines are mutated at this locus while only 12% of primary cutaneous melanomas are mutated (Hocker and Tsao 2007). In one study a mutation frequency of 30% of mucosal melanomas was found (Ragnarsson-Olding et al. 2002) suggesting subtype-specific mutation patterns. The low prevalence of p53 mutations in melanoma as compared to other cancers might be explained by the high frequency of *CDKN2A* inactivation. Evidence on the functional association between p14^{ARF} and p53 became clear when the interaction between Hdm2 and p14^{ARF} was elucidated (Zhang et al. 1998).

4.2

Somatic Changes in the Pigmentation Pathway of Melanoma

It was early demonstrated that the biology of pigmentation is an essential part of melanoma development. Several epidemiological studies demonstrate that dark skinned individuals have a decreased risk of developing melanoma and red-haired and light skinned people are at an increased risk of melanoma (Cho et al. 2005; Swerdlow et al. 1986). One of the key regulators of pigmentation is *MC1R* located on chromosome 16q and acts by inducing production of dark eumelanin over the red pheomelanin (Rouzaud et al. 2005). The dark eumelanin is thought to be more protective against UV radiation than the red pheomelanin. Genetic variants of the *MC1R* gene are, not unexpectedly, associated with an increased risk

of melanoma as reviewed elsewhere (Nelson and Tsao 2009). Also, genetic variants in other genes or loci (*TYR*, *ASIP*, *OCA2*, *TYRP1*, *SLC24A4*, *TPCN2*, and 9p21) connected to the pigmentation pathway have been shown to confer melanoma risk through a recent set of with genome-wide association (GWAS) studies (Bishop et al. 2009; Brown et al. 2008; Gudbjartsson et al. 2008). Although these GWAS results clearly link *MC1R* to risk, no *MC1R* somatic mutations were identified in a cohort of 103 primary melanomas suggesting that *MC1R* is not a frequent target of somatic alteration (Kim et al. 2008). In 2005, Garraway et al. used the NCI 60 panel of cell lines and an integrative genomics approach to map novel oncogenes. High-resolution genomic maps indicated lineage restricted copy number changes and integrated with gene expression profiling data-supervised methods identified *MITF* as amplified and highly expressed in a subset of melanomas (Garraway et al. 2005). It was at that time known that Mitf was a master regulator of the melanocyte lineage. When investigating *MITF* gene copy number status in a set of primary melanomas, an inverse relationship was observed between gene copy number and survival. Intriguingly, all *MITF*-amplified cell lines harbored *BRAF* mutation as well as inactivated p16^{INK4A} suggesting a cooperative effect of these genetic defects. Consequently, overexpression of *MITF* in combination with oncogenic *BRAF* in immortalized melanocytes transformed these thereby confirming the oncogenic potential of *MITF* in melanoma. Several major pathways such as normal pigment cell physiology, melanocyte survival, cell cycle regulation, and growth are known to be downstream activities of Mitf. An elegant study by McGill et al. showed that *BCL2* is a transcriptional target of Mitf confirming the growth regulatory function of Mitf since Bcl2 is an antiapoptotic factor (McGill et al. 2002). Moreover, additional targets of Mitf include Tbx2, Met, and Cdk2 representing mechanisms that provide growth advantage (Carreira et al. 2000; Du et al. 2004; McGill et al. 2006). The regulation of Mitf has been investigated and regulatory pathways include Notch, Mc1r, Wnt, endothelin receptor, KIT, and MAPK pathways. These pathways are known to genetically interact with Mitf, such as PAX3 and SOX10, and are involved in neural crest development (Potterf et al. 2000; Verastegui et al. 2000) (see Chap. 2 and 4). The critical function of Mitf in melanoma as well as the finding of *MITF*-amplified melanomas led to the discovery of somatic mutations of *MITF* in a subset of melanomas. In the study by Cronin et al. eight cases of 50 metastatic melanoma lines were either amplified or mutated for *MITF*. In line with these results an additional three cases harbored mutation of the *SOX10* gene, an upstream regulator of *MITF*, in a mutually exclusive fashion with *MITF* alterations (Cronin et al. 2009). Correlation between mutations in the Mitf pathway and *BRAF*/*NRAS* mutation status supported the observation made by Garraway et al. that these alterations are cooperative genetic events (Garraway et al. 2005). Overall, this study observed that approximately 20% of metastatic melanoma harbors genetic alterations of the Mitf pathway. Chromosome 7p gains/amplifications is commonly found in melanoma and with this in mind, Jane-Valbuena et al. set out to search for a potential novel oncogene in melanoma. Using high-density SNP arrays *ETV1* was pinpointed as an attractive target (Jane-Valbuena et al. 2010). Functional analysis revealed that the oncogenic potential of *ETV1* was dependent on concomitant activation of the MAPK pathway and upregulated mRNA levels of *MITF*. Additional studies of other members of the *MITF* pathway will most likely unravel additional gene alterations.

As an initiator of several melanocytic signaling pathways *KIT* has a central role in the development of melanoma. It has been demonstrated that *KIT* mutations in melanoma are mutually exclusive with *BRAF* and *NRAS* mutations. c-kit is a receptor tyrosine kinase and

has been proven to be able to activate both the MAPK and PI3K pathways (Fig. 4.1). Particular high frequency of mutations was identified in melanomas arising on palms, soles, and subungual sites (acral melanomas) as well as on mucosal membranes (mucosal melanomas) (Curtin et al. 2006). Additionally, gain or amplification of the *KIT* locus (chromosome 4q12) was observed preferentially in mucosal, acral, or chronically sun-damaged melanomas and a substantial number of mutated tumors also harbored amplification suggesting an additive effect of amplification and mutation. It remains to be elucidated whether the mutated allele is preferentially amplified.

Like *KIT*, β -catenin is also involved in melanocyte lineage development. Many studies have shown that β -catenin is critical for the transformation of melanocytes. Increased level of nuclear β -catenin is a consequence of a cascade initially started by canonical Wnt signaling and has been found in 50% of melanomas (Kielhorn et al. 2003). In mice, functional analysis has revealed that β -catenin cooperates with *NRAS* oncogenesis by repressing *p16INK4A* (Delmas et al. 2007). However, somatic mutations of β -catenin are detected in only a small fraction of melanomas suggesting that there are other unknown mechanisms responsible for the localization of β -catenin in the nucleus.

4.3

Novel Pathways in Melanoma

To identify novel key regulators of melanoma oncogenesis and metastasis several approaches have been taken where an integrative approach combining gene expression profiling and SNP arrays identified *MITF* as lineage-specific oncogene in melanoma as discussed previously. An alternative approach employed by Kim et al. includes comparative oncogenomics using a Ras inducible nonmetastatic mouse model to identify clones that had accumulated genetic changes conferring metastatic capacity (Kim et al. 2006). With this approach a focal amplification on mouse chromosome 13 containing eight genes was identified using high-resolution comparative genomic hybridization. When analyzing mRNA transcript levels *NEDD9* was pinpointed as the target gene. To confirm the results found in the mouse model a number of primary and metastatic melanomas were genome-wide screened for copy number changes. In all, 36% of metastatic and 8% of primary melanomas harbored amplification of 6p25-p24 (syntenic to mouse chromosome 13) as well as increased gene and protein expression of *NEDD9*. Furthermore, knockdown of *NEDD9* inhibited proliferation and invasion corroborating the metastatic capacity of *NEDD9*-activated melanoma cells.

A slightly different approach was employed by the group of Dr. Yardena Samules that recently performed a systematic genetic screen of the matrix metalloproteinase (MMP) superfamily of genes (Palavalli et al. 2009). Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade the basement membrane and the extracellular matrix. This suggests an involvement of MMPs in cancer metastasis; however clinical trials using MMP inhibitors are largely unsuccessful in clinical trials.

MMP8-deficient mice, however, exhibit an increased skin cancer risk suggesting a tumor suppressor role of *MMP8* in skin cancers including melanoma (Lopez-Otin et al. 2009). Notably, *MMP8* and *MMP27* were mutated in 6–8%, respectively, of the melanoma

cases investigated. Furthermore, mutations of *MMP8* were frequently accompanied by LOH suggesting that *MMP8* acts as a tumor suppressor gene in melanoma. Functional analysis of *MMP8* mutant cells displayed decreased proliferation as compared to *MMP8* wild-type cells. In contrast, when investigating migratory capacity of *MMP8* mutant cells these displayed decreased migration ability. Taken together, MMPs seem to play some role in melanoma development.

4.4

Genome-Wide Screening Approaches in Melanoma

Recent technical development has allowed researchers to investigate gene copy number changes on a global basis. In melanoma, availability of frozen tissue is a major drawback when it comes to genome-wide analyses; hence many studies in melanoma have been performed with melanoma cell lines or short-term cultures. Several research groups have investigated the detailed landscape of gene copy number changes in melanoma lines (Gast et al. 2010; Jonsson et al. 2007; Stark and Hayward 2007). Interestingly, a fairly homogeneous pattern of DNA copy number changes was observed with several chromosomes altered in more than 50% of the cases. Frequent losses were observed on chromosomes 4, 6, 8, 9, 10, and 11, whereas frequent gains were found on chromosomes 1, 7, 8, 17, and 20. These regions are corroborated by high-density SNP array analysis with frequent LOH at these regions (Stark and Hayward 2007). Additionally, 11q13, 3p14, and 1p12 were recurrently amplified including candidate oncogenes such as *CCND1*, *MITF*, and *NOTCH2*. Homozygous deletions are frequently identified in melanoma lines with the *CDKN2A* locus being affected in 40–60% and *PTEN* in 10–15%. Moreover, less frequent deletions include genes such as *PTPRD*, *HDAC4*, and *PARD3* suggesting novel tumor suppressor genes in melanoma.

In 2005, a vanguard study described genome-wide assessment of DNA copy number changes in 126 primary melanomas (Curtin et al. 2005). Tumors were divided into four groups based on sun-induced damage and on whether the tumor was classified as acral or mucosal melanoma. Specifically, acral and mucosal melanomas were found to harbor an increased frequency of genomic alterations as well as higher frequency of gene amplifications. Furthermore, chromosome 10q deletions were more common in melanomas without chronic sun-damaged skin, whereas focal gains of the *CCND1* locus were more frequent in the group with chronic sun-damaged skin. This elegant analysis of genetic alterations displayed that subgroups can be identified in melanoma and a strong relationship between sun exposure and genome-wide DNA copy number patterns exist.

More recently, primary and metastatic melanomas have been subjected to transcriptomic analysis. Using supervised and integrative methods a number of genes with a significant expression deregulation between primary and metastatic melanoma were identified. The proinvasive ability of these genes was subsequently validated with functional assays (Kabbarah et al. 2010).

In summary, during the past few years there have been an explosion of genomic analyses in melanoma. The heterogeneous nature of melanoma is clearly reflected in genome-wide DNA copy number changes suggesting several distinct genetic entrée into melanoma.

4.5

Progression of Melanoma: The Genomic Approach

The classical histopathological pathway of progression described by Clark is initiated in the benign nevus where proliferation of normal melanocytes begins. The next step includes the formation of dysplastic nevi and this may occur in pre-existing nevi or at a new location. The formation of radial growth phase melanomas occurs when melanocytes acquire the ability to proliferate intraepidermally. One of the most critical steps in the development of melanoma includes the progression from radially growing melanomas (i.e., radial growth phase, RGP) to vertically advancing melanomas (i.e., vertical growth phase, VGP) where cells have the ability to invade the dermis. This deep invasion allows melanoma cells to spread to distant organs, form metastases, and potentially create a lethal event. Dissemination can occur by either lymphatic or hematogenous routes. Since access to these vascular structures is a prerequisite for metastatic spread, it is not surprising that a simple measure of vertical invasion, i.e., Breslow thickness, has survived the test of time.

The genetic factors related to this histopathological progression are starting to emerge. The presence of somatic *BRAF* mutations in nevi (Pollock et al. 2003) suggests that this is an early event and that *BRAF* activation alone is not sufficient for melanocytes to transform into a malignant state. Subsequent genetic events are more uncertain though p16^{INK4A} loss is considered to be an important early step since germline mutations in *CDKN2A* have been found in a significant fraction of melanoma kindreds and homozygous deletions and deleterious mutations of *CDKN2A* occur in a large fraction of melanoma cell lines. Also, it is evident that mice with *Cdkn2a* loss cooperate with activating MAPK lesions to drive melanoma formation (Chin et al. 1997). In addition to *CDKN2A*, loss of *PTEN* should also be considered to be a critical event. This is supported by human studies where deleterious *PTEN* alterations have been described (Tsao et al. 2004) and also by mice studies where *Pten* loss has been shown to induce metastatic melanoma in combination with activating *Braf* alleles (Dankort et al. 2009).

Beyond discrete genetic events, markers that define the switch from RGP to VGP have also been examined. One of the hallmarks in the switch between RGP and VGP include the loss of E-cadherin expression and simultaneously increased expression of N-cadherin affecting cell adhesion mechanisms. In addition, these expression differences stimulate β -catenin and thereby increase the survival of melanoma cells. In a more comprehensive screen, Haqq et al. investigated gene expression difference between RGP and VGP (Haqq et al. 2005). Interestingly, loss of expression for a set of genes was observed in the vertical growth phase of the melanoma; these include *CDH3* and *MMP10* among other genes. Furthermore, the same gene set effectively partitions metastatic melanomas into two groups, which might indicate that metastases could develop from either the radial growth or the vertical growth phase of melanoma. The authors also identified gene sets that differed between normal skin, nevi, primary melanoma, and two types of metastatic melanoma suggesting that molecular patterns of progression do exist. Likewise, Sabatino et al. characterized melanoma cell lines derived from metachronous metastases from a single melanoma patient (Sabatino et al. 2008). Genome-wide copy number analysis supported a cancer stem cell model where a suggestive progenitor cell harbors a core set of genomic aberrations and that metastatic progression is not a sequential event. In a similar study by Harbst

et al. metastases obtained from five patients were investigated for genomic imbalances, gene expression profiles, and methylation profiles (Harbst et al. 2010). Here, evidence for two models of metastatic progression was provided. The first model was supported by genomic aberration patterns of a case with three metachronous metastases. Homozygous deletions at chromosomes 3p26 and 6q23 were found in two consecutive metastases originating from the same primary tumor in a mutually exclusive manner corroborating the findings of Sabatino et al. (Sabatino et al. 2008). Investigation of the E-cadherin and N-cadherin mRNA levels indicates variability as to when the actual switch in expression occurs. For one case the switch had obviously occurred prior to the metastases investigated, whereas in two of the cases the switch became apparent at the development of the second metastasis (Fig. 4.2). Interestingly, methylation analysis provided additional

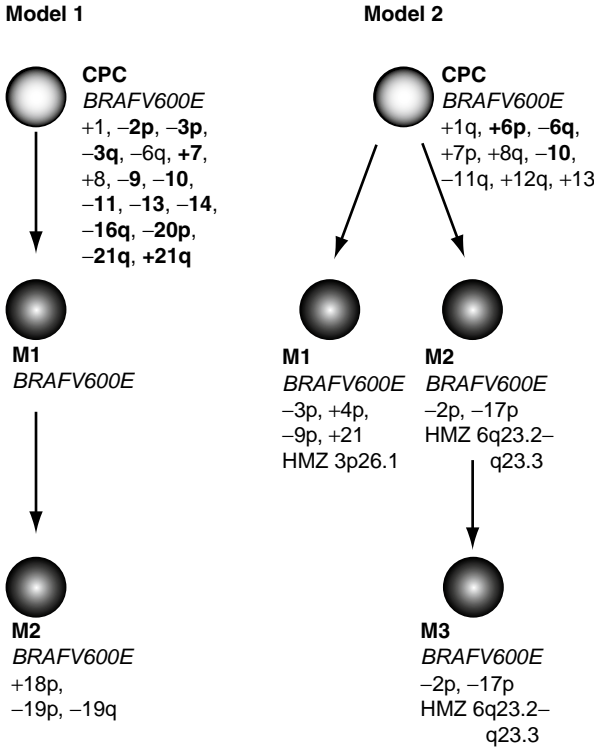


Fig. 4.2 Two models of tumor progression as suggested by Harbst et al. (2010). *Model 1*: The indistinguishable genomic profiles, as well as inheritance of the *BRAF* mutation, suggest sequential tumor progression. *Model 2*: Metastasis-specific gene copy number aberrations exclude the possibility of a sequential process and indicate to the evolution of at least two distinct subclones with metastatic potential. However, both a core set of genomic aberrations and presence of the *BRAF* mutation in all lesions confirm common origin of the tumors. Major chromosomal changes are indicated. CPC indicates a common metastatic precursor cell/clone that harbors indicated chromosomal aberrations inherited by all the metastases from that tumor. In bold, copy number aberrations observed in the primary tumor. HMZ, homozygous deletion

evidence for *PTEN*'s role in melanoma progression. Silencing of the *PTEN* gene was evident in the last of three consecutive metastases originating from a single primary melanoma. An alternative model of sequential progression was also found. In another case, the primary tumor as well as two consecutive metastases displayed almost indistinguishable genomic profiles. Furthermore, gene expression and methylation profiles support a common origin for metastases originating from the same primary tumor. In all, this indicates that at least two types of mechanisms can contribute to melanoma metastases. These recent studies where a genomic approach was taken indicates the extensive complexity in progression of malignant melanoma and more in-depth genetic studies are eagerly awaited.

4.6

Molecular Classification of Melanoma

Several groups have used molecular parameters in an attempt to recover a subclassification of melanoma. Viros et al. used a combination of histomorphologic and genetic features to classify a comprehensive set of melanomas (Viros et al. 2008). Interestingly, *BRAF*-mutated melanomas displayed characteristic features such as nest formation of intraepidermal melanocytes and larger, rounder, and more pigmented tumor cells. In contrast, *NRAS*-mutated melanomas did not show any specific morphologic characteristics. A survival benefit was also identified in patients predicted to be *BRAF* mutated, most likely due to a different route of metastasis. As mentioned previously, Curtin et al. described a classification based on genetic alterations and primary tumor location and sun-damaged tumor surrounding skin (Curtin et al. 2005). A more recent study also used genome-wide DNA copy number changes as a way of subclassifying melanoma metastases. Here, three classes were found and showed different event-free survival (Kabbarah et al. 2010). An alternative way of molecularly classifying cancer is by using genome-wide assessment of mRNA transcript levels though the lack of frozen tissue from primary melanomas has led to only one study with sufficient clinical annotation (Kabbarah et al. 2010). In that study, 83 primary melanomas in the vertical growth phase with a Breslow thickness of at least 1 mm were subjected to global gene expression profiling. Applying supervised methods on melanomas from patients with or without 4 year distant metastasis-free survival a prognostic set of 254 genes was identified. Investigation of clinical parameters in the two prognostic groups revealed differences with regard to Breslow thickness, AJCC stage, melanoma type, ulceration, and mitotic rate – features all known to be strong prognostic factors in melanoma. Indeed, comparison of the prognostic gene set and a gene set derived based on Breslow thickness revealed a considerable overlap. Genes related to DNA repair and cell cycle increased with thickness as well as with poor prognosis. A more careful analysis of the prognostic gene set showed that DNA replication and DNA repair are the most significant pathways (Kauffmann et al. 2008). These results have also been corroborated by a recent study applying an alternative microarray platform (Jewell et al. 2010).

Technical developments have enabled researchers to start exploring global gene expression profiles from formalin-fixed paraffin-embedded (FFPE) tissue (April et al. 2009). In

melanoma, the first studies used a microarray platform consisting of a restricted set of cancer-related genes ($n=502$) (Conway et al. 2009; Jewell et al. 2010; Mitra et al. 2010). However, these have been directed toward identifying single molecular biomarkers and not in classifying melanomas. Through such approaches, the expression level of osteopontin was identified as the gene with strongest association with reduced relapse-free survival (Conway et al. 2009). Subsequent studies with sentinel lymph node status confirmed the prognostic significance of osteopontin and DNA repair genes (Jewell et al. 2010; Mitra et al. 2010). Studies using whole-genome approaches of gene expression analysis of archival FFPE RNA open up a wide range of opportunities for investigating the genomic landscape of primary malignant melanoma.

Global gene expression patterns have to a larger extent been applied to advanced stage or metastatic melanomas where availability of frozen tissue is not the main limitation. As alluded to earlier, one study used a gene set derived from a discriminatory analysis between radial growth and vertical growth phase of a single primary melanoma (Haqq et al. 2005). In this study, the investigators also detected two subtypes of metastatic melanoma. Type I metastases displayed increased expression of genes presumably downregulated in the vertical growth phase of primary melanoma, whereas type II metastases were characterized by increased expression of genes related to pigmentation such as *MITF*, *MLANA*, and *TYR*. More recently, Jönsson et al. set out to discern a biological subclassification with clinical impact in stage IV melanoma (Jonsson et al. 2010). Gene expression profiles were obtained for 57 lymph node or subcutaneous metastases. Unsupervised hierarchical clustering algorithms identified four molecular classes. These were subsequently characterized and named according to the gene expression pattern of the class. The first group, high-immune response, expressed high levels of immune response-related genes such as *LCK*, *CXCL12*, and *HLA class I and II antigen*. The second class, proliferative, expressed decreased levels of immune response-related genes as well as a trend toward higher Ki67 staining. High levels of genes such as *MITF*, *TYR*, *DCT*, and *MLANA* characterized the third class (Pigmentation class). This is in line with the findings of Haqq et al. who identified a subclass in metastatic melanoma that had high expression of genes that characterize the radial growth phase of a single primary melanoma as well as high expression of pigmentation-related genes (Haqq et al. 2005). Finally, the normal-like subclass was characterized by melanomas expressing genes such as *TRIM29*, *KRT17*, and *KRT10*. Most importantly, this biological subclassification scheme displayed a significant association to clinical outcome with the proliferative subtype as a poor prognostic group, which was validated in an independent cohort of stage III melanomas (Fig. 4.3). In a study by Bugonvic et al. a prognostic gene expression signature supports the finding of improved survival in patients with tumors having increased expression of immune response-related genes (Bogunovic et al. 2009). Additionally, all patients included in the Jönsson et al. study were enrolled in a prospective trial of DTIC treatment. This allowed the investigators to correlate treatment response with molecular subclass. Indeed, a significant association between the pigmentation subclass and a stable disease at 3 months of treatment was identified suggesting that pigmentation might be a predictive target of DTIC response. Further molecular characterization of subclasses implied different frequencies of genomic imbalances and targeted gene deletions, e.g., *CDKN2A* homozygous deletion was significantly associated with the proliferative class. In all, this study indicates that a molecular classification is tenable and could be clinically meaningful.

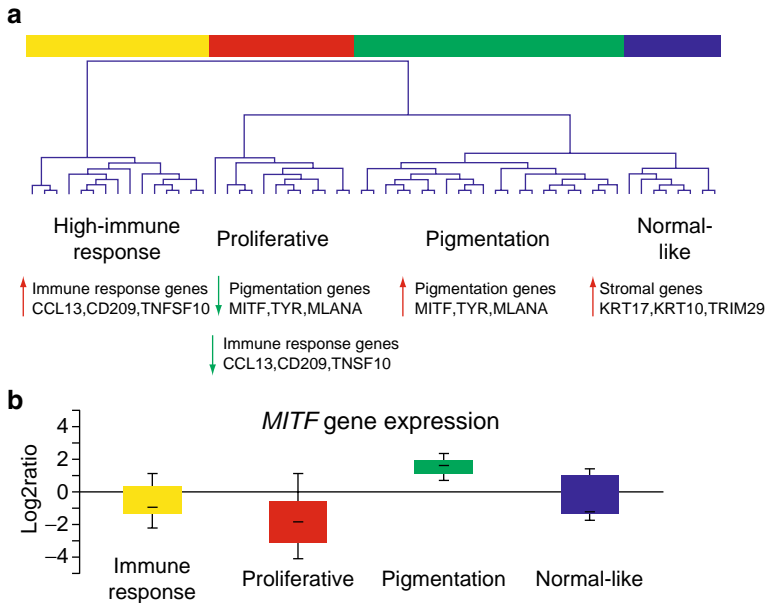


Fig. 4.3 Molecular classification of malignant melanoma. (a) Subclassification of stage IV melanomas based on gene expression patterns as suggested by Jönsson et al. (Jonsson et al. 2010). Four classes were identified each broadly characterized by expression of set of genes involved in immune response and pigmentation. (b) Boxplot of *MITF* gene expression levels in each class displaying an increased expression in the Pigmentation class

In summary, classification of melanoma based on molecular patterns will most likely be an important parameter in future medical oncology since the advent of targeted therapy showing promising results in melanoma will require molecular stratification prior to treatment.

4.7 Next-Generation Sequencing: Approaches in Melanoma

The availability of novel high-throughput sequencing methods such as human genome sequencing has revolutionized cancer research during the past few years. In a pioneering study from the Wellcome Trust Sanger Institute, a single melanoma cancer genome was sequenced with a tremendous gain in biologic insight (Plesance et al. 2010). One of the main findings of the study was the observation that the melanoma genome was littered with C>T mutations 3' to a pyrimidine site, a hallmark of mutations attributed by UV-induced DNA damage. This single finding puts to rest the controversy that melanomas are in fact not subject to UV mutagenesis (Hocker and Tsao 2007) (see also Chap. 3). It is also possible to sequence RNA transcripts using next-generation sequencing. An elegant study by Berger et al. used this approach to analyze short-term melanoma cultures

(Berger et al. 2010). In the analysis, 11 novel melanoma gene fusions produced by underlying genomic rearrangements were found. However, none of these changes were recurrent suggesting that the gene fusions could be “passenger” events. Interestingly, in a screen for novel prostate cancer fusion genes, *BRAF* was identified and because of the high mutation rate of somatic mutations of *BRAF* in melanoma the screen was extended to include melanomas; however, only one case out of 131 harbored a rearranged *BRAF* gene corroborating the low prevalence of recurrent gene fusions in melanoma (Palanisamy et al. 2010).

A very important study demonstrating the significance and enormous potential of next-generation sequencing was published in 2009 (Emery et al. 2009). Here, a somatic *MEK* mutation was observed in a metastatic lesion showing resistance to treatment of a *MEK* inhibitor. *In vitro* analysis displaying further evidence of *MEK*-mutated clones demonstrated resistance to both a *MEK* inhibitor as well as to a *Raf* inhibitor. However, when combining *MEK* and *Raf* inhibitors resistance to targeted therapies of the *MAPK* pathway is circumvented.

4.8 Summary

In summary, the genetic and genomic landscape of melanoma has radically changed over the past decade. Advances in our understanding of cancer genetics and the advent of powerful technologies will undoubtedly transform our vision of the molecular underpinnings of melanoma within the next few years. In this chapter, we have been able to survey only some of the principles and technologies that underlie the ongoing genomic revolution. It is clear that only the tip of the iceberg has come into view.

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Abstract Melanoma is one of the most chemo-resistant cancers and one whose incidence is increasing significantly. Transcription factors are DNA-binding proteins which control gene expression through recognition of specific DNA sequences within target promoter or enhancer regions, either directly or through protein–protein interactions. Many transcription factors have been shown to be oncogenes or tumor suppressors in melanoma. Some of them exhibit activities thought to be common to many cancers, whereas others are thought to be specific to melanoma or melanocytes. Each transcription factor modulates biological activities through a multitude of target genes and in many cases the transcription factor’s activity may be modulated by post-translational modifications. Unlike enzymes or receptors, only a small fraction of transcription factors have been successfully targeted for therapeutic purposes by small molecules, the most striking examples being nuclear hormone receptors which are transcription factors that are dependent upon binding and activation by drug-like nuclear hormones (e.g., sex steroids, glucocorticoids, etc.). Understanding the regulatory mechanisms and target genes of biologically important transcription factors could lead to the identity of drugable pathways for melanoma. This is particularly important if the spectrum of currently druggable oncoproteins is insufficient to produce curative outcomes or if transcriptional mechanisms contribute importantly to resistance from other targeted therapeutic approaches. In this chapter, we select a number of transcription factors that have been implicated in melanoma biology (MITF, CREB, SOX10, PAX3, Snail superfamily, FOXD3, Ets family, Brn2, AP-1, AP-2, LEF/TCF/ β -catenin, Notch, NF- κ B, SMAD/SKL, STAT3, HIF1A, Tbx-2/3, C-MYC, and p53) and focus on their regulatory mechanisms and biological targets.

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5.1

MITF (Microphthalmia-Associated Transcription Factor)

MITF is a basic helix-loop-helix leucine zipper protein, which binds to E-box sequences (5'-CA(C/T)GTG-3') as a homo- or heterodimer with other MiT family members (itself, TFE3, TFEB, or TFEC) (Hemesath et al. 1994; Bentley et al. 1994; Yasumoto et al. 1994). MITF has many isoforms, which are transcribed from different initial exons and exhibit tissue-specific expression patterns depending upon promoter usage (Hershey and Fisher 2005). Among isoforms, MITF-M, with its exon1M, is highly expressed in melanocyte-lineage cells (Fuse et al. 1996).

MITF has been shown to be required for melanocyte development, differentiation, and survival (see also Chap. 2). Mutations of MITF in humans cause Waardenburg syndrome (WS) 2A, which is an autosomal dominant auditory-pigmentary syndrome characterized by pigmentary abnormalities of the hair, skin, and eyes and congenital sensorineural hearing loss (Tassabehji et al. 1994). Appropriate regulation of MITF is required for cell growth/survival in melanocytes and recent work has implicated MITF as a genomically amplified oncogene in 10–20% of human melanomas (Garraway et al. 2005). In addition, human Clear Cell Sarcoma is a frequently pigmented soft tissue sarcoma which harbors a chromosomal translocation producing the EWS-ATF1 fusion protein (Davis et al. 2006). This chimeric oncoprotein is thought to mimic the normally cAMP-regulated ability of ATF1 to activate M-MITF expression, instead of constitutively activating the *M-MITF* promoter. The result is dysregulated M-MITF expression in Clear Cell Sarcoma which likely accounts for the tumor's melanin production as well as oncogenic behavior (Davis et al. 2006). In addition, ~ 20% of metastatic melanomas were found to harbor somatic coding mutations, which may represent an additional oncogenic mechanism (Cronin et al. 2009).

MITF transactivates multiple genes related to cell cycle (CDK2, p16, p21, and Tbx2), pigmentation/differentiation (TYR, TYRP1, DCT, MART1, AIM-1, TRPM1, and PMEL17), apoptosis (HIF1A, BCL2), and motility (c-MET) (Fig. 5.1).

MITF transcription is regulated by other transcription factors, including PAX3, SOX10, CREB, FOXD3, LEF-1, and ONECUT-2. CREB/ATF1-mediated regulation of MITF is important for physiological expression in melanocytes, under the control of cAMP or other signals. MITF is also post-translationally regulated by phosphorylation (via MAPK, GSK-3b, and RSK) (Weilbaecher et al. 2001; Wu et al. 2000; Takeda et al. 2000; Mansky et al. 2002), sumoylation (Miller et al. 2005; Murakami and Arnheiter 2005), caspase-dependent cleavage (Larribere et al. 2005), and ubiquitination. The MAPK-phosphorylated MITF is ubiquitinated and degraded via the proteasome pathway. MITF activity can be suppressed by overexpression of PIAS3, which directly binds to MITF and inhibits DNA binding (Levy et al. 2002).

Recent work has indicated that nonspecific histone deacetylase inhibitors (HDACi) could potently repress MITF expression in melanoma cells. Suppressed MITF expression and cell growth were produced in melanoma cell lines and in mouse xenografts in response to treatment with a variety of HDACi (Yokoyama et al. 2008).

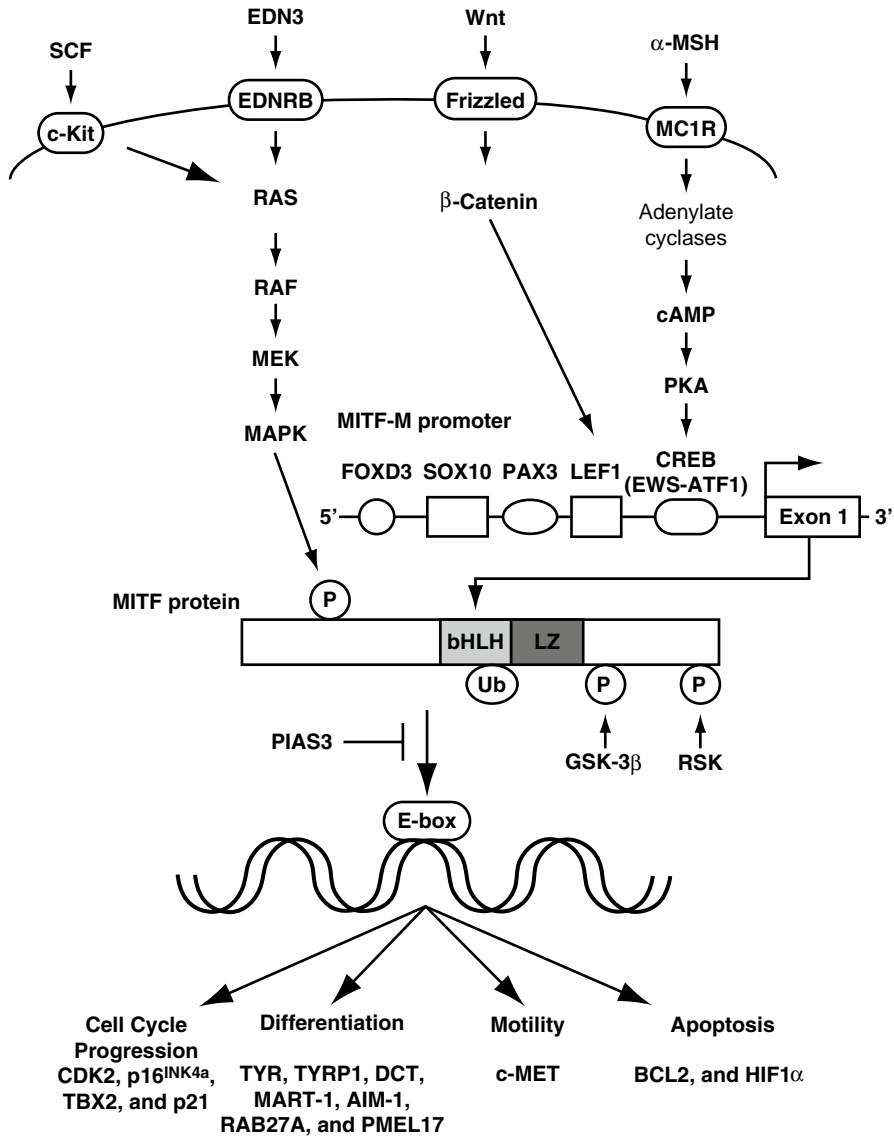


Fig. 5.1 MITF is involved in the induction of melanoma, melanocyte differentiation, cell-cycle progression, and survival. Black lines represent some of the signaling pathways connected to MITF, such as steel factor (*SCF*) and endothelin-3 (*EDN3*), and α -melanocyte stimulating hormone (*α -MSH*). White circles and squares represent transcription factor-binding sites on the MITF-M promoter. MITF protein is post-translationally regulated by phosphorylation via MAPK, GSK-3 β , and RSK. PIAS3 suppresses the DNA-binding of MITF. MITF protein binds to consensus sequences, a subset of E-boxes, present in promoter or enhancer elements containing the consensus CATGTG or CACGTG. MITF regulates multiple targets in melanocytes and melanoma cells involved in various cellular processes such as cell-cycle control, survival, motility, invasion, differentiation, and/or pigmentation

5.2

cAMP-Responsive Element (CRE)-Binding Protein/Activating Transcription Factor-1 (CREB/ATF-1)

CREB/ATF-1 is one of the most important transcription factors in G-protein coupled receptor (GPCR) signaling. In normal melanocytes, CREB/ATF-1 is required for pigmentation signaling from melanocortin receptor type1 (MC1R) to MITF, which plays a central role in pigmentation as mentioned above (Price et al. 1998). α -melanocyte stimulating hormone (α -MSH) is produced after UV exposure in human skin (Lin and Fisher 2007; Yamaguchi and Hearing 2009); then bound to MC1R, which is a GPCR, in melanocytes. MC1R activates protein kinase A via cAMP production. Activated PKA phosphorylates CREB at Ser133, which induces transcription activity via a highly conserved CREB-binding site (cAMP-responsive element; CRE) (5'-TGANNTCA-3') in the *M-MITF* promoter. CREB1 also regulates somatostatin, proenkephalin, fos, and cyclin D1 (Barr et al. 1999).

EWS-ATF1 fusion protein plays an oncogenic role in human clear cell sarcoma via MITF (Davis et al. 2006). It is reported that the transition of melanoma cells from radial to vertical growth phase is associated with overexpression of CREB/ATF-1. Dominant-negative CREB (KCREB) can decrease melanoma tumorigenicity (possibly via MITF) and metastatic potential (via MMP-2 and MCAM/MUC18) in nude mice (Xie et al. 1997). Taken together, CREB/ATF1 contributes importantly to melanoma behavior.

5.3

SOX (Sry-Box) Family (SOX9 and SOX10)

The SOX family is comprised of approximately 20 transcription factors named after the original member, Sry (sex-determining region Y), because they all share a similar high-mobility-group (HMG) domain (Soullier et al. 1999) which recognizes the consensus sequence (5'-(A/T)(A/T)CAA(A/T)G-3'). SOX proteins bind DNA as a monomer or a dimer, and then regulate the transcription of target genes (Jiao et al. 2004; Ludwig et al. 2004; Peirano and Wegner 2000).

Among SOX family members, SOX10 is one of the major players in melanocyte development, and some recent data have suggested a significant role for SOX9 as well. In humans, SOX10 mutation causes WS type 4, which is characterized by sensorineural deafness/hypopigmentation associated with WS and enteric aganglionosis associated with Hirschprung's disease (Herbarth et al. 1998; Southard-Smith et al. 1998). SOX8 and SOX9 play roles in neural crest development, which is the origin of melanocytes (Cheung and Briscoe 2003; Maka et al. 2005; Spokony et al. 2002).

UV exposure induces SOX9 via a cAMP/PKC-dependent pathway (Passeron et al. 2007) and SOX9 increases MITF and DCT expression in a SOX10-independent manner. In the case of SOX10, there is little information about its own transcriptional regulation. The tyrosine kinase TYRO3 can control SOX10 nuclear localization, resulting in

subsequent SOX10-dependent increases in MITF, together with melanoma cell proliferation and survival (Zhu et al. 2009).

SOX10 regulates MITF, EDNRB, and c-ret, which are also related to WS and Hirschprung's disease. The function of SOX10 in melanomagenesis is still unknown, but recently SOX10 somatic mutations were reported in ~7% of metastatic melanomas (Cronin et al. 2009).

5.4

PAX3 (Paired Box 3)

PAX proteins represent a highly conserved family of transcription factors required for the development of multiple tissue types. Pax3 is a particularly interesting member of the Pax family, which coordinates the development of certain neural crest-derived lineages including skeletal muscle and melanocytes (Tassabehji et al. 1993). In humans, specific PAX3 mutations may cause either WS type 1 or type 3 characterized by abnormalities of the central nervous system, face, eye, nose, cochlea, and hair pigmentation.

PAX3 has two DNA binding domains: a paired domain and a homeodomain (Goulding et al. 1991). The paired domain binds the consensus sequence (5'-GT(T/C)(C/A)(C/T)(G/C)(G/C)-3') whereas the homeodomain recognizes DNA containing a core sequence (5'-TAAT-3'). Pax3 can serve as a transcriptional activator or repressor by binding different partner proteins, such as SOX10 and TAZ (as activators) and TLE4 (Grg4 in mice), KAP1, and HP1 γ (as repressors) (Bondurand et al. 2000; Hsieh et al. 2006; Lang et al. 2005; Murakami et al. 2006).

As predicted from WS types1/3, PAX3 regulates MITF, c-Ret, WNT1, and TGF- β 2, which are required for the development of neural crest derivatives. PAX3 also regulates pigmentation genes (DCT and TRP1), anti-apoptotic genes (PTEN, BCL-XL), and differentiation-related genes (FGFR4, C-MET, MYF-5, MyoD, MSX2, HES1, and NGN2).

Pax3's precise function in melanoma is not known, but PAX3-FOXO1 fusion protein is found in Alveolar rhabdomyosarcoma (aRMS) (Barr et al. 1993; Galili et al. 1993). Knockdown of PAX3-FOXO1 transcripts in aRMS cells produces reduced cell motility, allows differentiation to proceed, and decreases proliferation rates (Kikuchi et al. 2008). PAX3 overexpression is observed in many primary melanomas, melanoma cell lines, and melanoma sections (Barr et al. 1999; Medic and Ziman 2010; Plummer et al. 2008; Scholl et al. 2001). These data suggest that PAX3 might play an important role in melanomagenesis or tumor maintenance.

5.5

Snail Superfamily (Snail and Slug)

Snail family members encode transcription factors of the zinc-finger type (Boulay et al. 1987). They share a similar organization, being composed of a highly conserved carboxy-terminal region, which contains 4–6 zinc fingers, and a more divergent amino-terminal region. The fingers are of the C2H2 type and function as sequence-specific DNA-binding motifs (Knight

and Shimeld 2001). The consensus binding site for Snail-related genes contains a core of six bases, 5'-CAGGTG-3' (Mauhin et al. 1993), which also corresponds to a subset of the E-box motif (5'-CANNTG-3'). On binding to their E-boxes, Snail family members act as transcriptional repressors (Batlle et al. 2000; Cano et al. 2000). In humans, mutation of SLUG causes WS type 2D, which is characterized by an auditory-pigmentary syndrome characterized by pigmentary abnormalities of the hair, skin, and eyes and congenital sensorineural hearing loss (Sánchez-Martín et al. 2002). SLUG was also identified as an MITF target gene.

In cancer, SNAIL/SLUG is related to the epithelial–mesenchymal transition (EMT), which is thought to be a key step in cancer metastasis (Batlle et al. 2000; Cano et al. 2000). Activation of Snail expression plays an important role in downregulation of E-cadherin, contributing to tumorigenesis of malignant melanomas (Poser et al. 2001). *In vitro* and *in vivo* experiments suggest that Slug is required for the metastasis of the transformed melanoma cells (Gupta et al. 2005). SNAIL/SLUG downregulates EMT-related genes, such as E-cadherin, Claudins, and Occludin.

5.6

FOXD3 (Forkhead Box D3)

FOX transcription factors represent a closely related family of proteins that mediate cell-cycle progression, survival, and differentiation (Myatt and Lam 2007). The FOX transcription factors contain a conserved FOX or winged helix domain, which is required for binding to a consensus DNA sequence and for activating target gene transcription.

Foxd3 acts as transcriptional repressor via a consensus binding site (5'-A(A/T)T(A/G)TTTGT-3') and is expressed in migratory neural crest (NC) cells that give rise to multiple lineages. Kos et al. (2001) showed that experimental downregulation of FOXD3 results in an increase in the number of differentiating melanocytes in quail NC cultures, and in premature dorsolateral migration of chick NC cells. Conversely, misexpression of FOXD3 in melanoblasts results in a failure of NC cells to enter the dorsolateral pathway. Ectopic expression of FOXD3 represses MITF in cultured NC cells and in B16-F10 melanoma cells via inhibition of PAX3 binding to MITF promoter (Thomas and Erickson 2009).

As FOXD3 can regulate MITF, which is an oncogene in human melanoma, FOXD3 is thought to prevent melanoma growth. Recently, it was reported that B-RAF^{V600E}, which is one of the major mutations in melanoma, suppresses FOXD3 levels in human melanoma cells and FOXD3 expression represses melanoma growth by inhibiting the G1-S transition (Abel and Aplin 2010). These data support FOXD3 as a suppressor of melanoma growth.

5.7

ETS Family Members

ETS transcription factors have a conserved DNA-binding domain (the ETS domain) of about 85 amino acids. The ETS domain, which bears a winged helix-turn-helix protein fold, mediates binding to a core DNA sequence (5'-GGA(A/T)-3'), with adjacent sequences

influencing binding affinities (Sharrocks 2001). ETS transcription factors are important in many biological settings such as cell growth, differentiation and survival, and in processes that include hematopoiesis, angiogenesis, wound healing, cancer, and inflammation. At least 27 ETS family members have been described in mammalian cells and nearly two-thirds are ubiquitously expressed in adult tissues.

Multiple members of the ETS family undergo oncogenic dysregulation in cancer, often through chromosomal translocation. In Ewing's sarcoma (EWS), EWS-ETV1 translocations result in highly transforming chimeric ETS fusion proteins (Bailly et al. 1994; Ouchida et al. 1995). Chromosomal translocations involving ETV1 and other ETS genes were found in more than 40% of prostate cancers (Tomlins et al. 2005). Most commonly, these translocations interpose the promoter and 5' coding exons of the *TMPRSS2* gene upstream of an ETS factor gene (*ERG*, *ETV1*, *ETV4*, or *ETV5*), resulting in androgen-dependent regulation and elevated expression of these genes.

In melanoma, translocations involving Ets family members have not yet been described. But recently amplification of the *ETV1* locus (>40%) and dependency on *ETV1* expression for melanoma proliferation were reported (Jané-Valbuena et al. 2010). The oncogenic activity of *ETV1* is thought to be mediated by another oncogene, *MITF*.

5.8

BRN2 (POU3F2/N-Oct-3)

POU domain transcription factors are present in many cell lineages where they perform varying functions, either as ubiquitous regulators of “house-keeping” genes, or as developmental- and lineage-specific coordinators of cell fate decisions. The POU domain is a highly conserved DNA binding structure, which was first found to recognize the canonical octameric sequence (5'-ATGCAAAT-3') (Pruijn et al. 1986, 1987). Later, two subdomains, POU_s and POU_h, were identified to bind to DNA sequences 5'-ATGC and 3'-AAAT, respectively (Sturm and Herr 1988). POU proteins are capable of homodimerization on DNA target binding sites and interaction with a variety of other proteins, such as SOX family members (Wegner 2005). A major interaction between the POU family and SOX family involves OCT4 (POU5F1) and SOX2 in embryonic stem cells (Yuan et al. 1995). BRN2, SOX10, SOX9, and PAX3 are expressed in melanocytes and physical interactions among SOX10, PAX3, and BRN2 have been reported (Smit et al. 2000). BRN2 acts as a repressor of *MITF* expression in melanoma cells via direct binding to a region adjacent to the TATA box, resulting in suppressing the differentiated melanocytic phenotype and enhancing tumor metastasis (Goodall et al. 2008).

5.9

AP-1 (Activator Protein 1)/ATF2 (Activating Transcription Factor 2)

The AP-1 transcription factor is a dimeric complex that comprises members of the JUN, FOS, ATF (activating transcription factor), and MAF (musculoaponeurotic fibrosarcoma) protein families (Vogt 2002). AP-1 proteins are known as basic leucine-zipper (bZIP)

proteins because they dimerize through a leucine-zipper motif and contain a basic domain for interaction with DNA. The AP-1 complex can form various different combinations of heterodimers or homodimers, and these combinations determine the genes that are regulated by AP-1. AP-1 upregulates transcription of genes containing the TPA response element (TRE; 5'-TGA(G/C)TCA-3').

ATF2 is well-characterized and thought to play a key role in melanoma (Bhoomik et al. 2007). ATF2 activity is regulated by phosphorylation on threonine (Thr) residues 69 and 71 via stress-activated kinases JNK, RalGDS-Src-P38 pathway, ATM, and Ras-MEK-ERK (Bhoomik et al. 2005; Gupta et al. 1995; Ouwers et al. 2002). Stimuli that activate these kinases, including exposure to proinflammatory cytokines, UV irradiation, DNA damage, or changes in ROS, are among the inducers of ATF2 transcriptional activity. Following phosphorylation, ATF2 activates transcription through heterodimerization with other transcription factors, among which c-Jun has been best characterized. ATF2/c-Jun heterodimers preferentially bind to the consensus sequence (5'-T(G/T)ACNTCA-3'). In addition to phosphorylation, ATF2 is also regulated by ubiquitin-dependent degradation by the proteasome, which is dependent on the association with JNK (Fuchs et al. 2000). ATF2 also functions in the DNA damage response by associating with the TIP60 HAT complex (Bhoomik et al. 2008). The TIP60 complex is required for ATM self-phosphorylation via acetylation. ATF2 regulates ATM activation by control of TIP60 stability and activity.

Through its dimerization with specific partners, ATF2 regulates expression of its transcriptional targets. These are stress/DNA damage response genes (c-Jun, c-fos, ATM, XPA, RAD23B, etc.), growth/tumorigenesis genes (cyclin A, cyclin D1, MMP2, TNF- α , etc.), and physiological homeostasis genes (tyrosine hydroxylase, collagen, VCAM-1, PGC-1 α , etc.).

There are several lines of striking evidence regarding ATF2 function in melanoma (Bhoomik et al. 2007). Nuclear ATF2 expression is a strong predictor of poor survival in melanoma patients (Berger et al. 2003) and interfering with ATF2 transcriptional activity can inhibit proliferation of melanoma cells in culture and formation of tumors and metastasis in mouse models. Taken together, ATF2 appears to act as an oncogene in melanoma.

5.10

AP-2

The AP-2 family of transcription factors consists in humans and mice of five members, AP-2a, AP-2b, AP-2c, AP-2d, and AP-2e (Hilger-Eversheim et al. 2000). All AP-2 proteins share a highly conserved helix-span-helix dimerization motif at the carboxyl terminus, followed by a central basic region and a less conserved domain rich in proline and glutamine at the amino terminus. The proteins are able to form hetero- as well as homodimers. The helix-span-helix motif together with the basic region mediates DNA binding, and the proline- and glutamine-rich region is responsible for transactivation. AP-2 has been shown to bind to the palindromic consensus sequence (5'-GCCNNNGGC-3') found in various cellular and viral enhancers. AP-2 activity is regulated through a number of signal transduction pathways. Phorbol esters and signals that enhance cAMP levels induce AP-2 activity independent of protein synthesis, whereas retinoic acid treatment of teratocarcinoma cell

lines results in transient induction of AP-2 mRNA levels at a transcription level (Buettner et al. 1993; Lüscher et al. 1989.).

Many experiments have demonstrated the functions of AP-2 in melanoma (Bar-Eli 2001). For example, loss of AP-2 expression increases tumor growth and metastasis in melanoma cells (Jean et al. 1998) and dominant-negative AP-2 (AP-2B) augments melanoma tumor growth *in vivo* (Gershenwald et al. 2001). AP-2 function is mediated via its regulated expression of target genes, which appear to be involved in proliferation, cell-cycle regulation (HER-2, p21/WAF-1), apoptosis (c-KIT, Bcl-2, FAS/APO-1), adhesion (MCAM/MUC18, E-cadherin), and invasion:angiogenesis (MMP-2, plasminogen activator inhibitor type I, VEGF, and PAR-1). Some of its targets are known by virtue of their deregulation upon loss of AP-2, suggesting that progression of human melanoma may be associated with loss of AP-2 expression.

5.11

LEF/TCF/ β -Catenin (Canonical Wnt Signaling)

LEF1/ β -catenin are downstream mediators of canonical Wnt signaling, and are involved in a variety of processes in development and tumorigenesis (see also Chap. 7). The Wnt family consists of over 19 members, all of which are hydrophobic cysteine-rich secreted molecules that share a high level of homology. The ligand subtype determines which Wnt signaling pathway will be activated. For example, Wnt1, 3a, and 7 activate the canonical pathway, whereas Wnt5a, 5b, and 11 activate the noncanonical pathway (Weeraratna 2005.). Receptors of Wnt ligands include the Frizzled (FZD) family of receptors (Zilberberg et al. 2004).

Wnt signaling plays a critical role in the development of the neural crest, specifically Wnt1 and Wnt3A (Dorsky et al. 1998; Dunn et al. 2000). Wnt1 and Wnt3A also promote the development of neural crest cells into pigment cells. When cells are depleted of these two proteins they become neuronal rather than pigment cells (Dunn et al. 2005).

The activation of the canonical Wnt pathway involves the multifunctional protein β -catenin. In the absence of Wnt, β -catenin is targeted to a multimeric protein complex called the destruction complex, where it becomes phosphorylated. This is achieved by CK1- or glycogen synthase kinase (GSK) 3 β -mediated phosphorylation. The phosphorylated β -catenin is ubiquitinated and degraded by the proteasome. In the presence of some Wnt ligands, a cascade of events initiated at the plasma membrane by the binding of Wnt to the cysteine-rich domain of Frizzled receptors results in the disassembly of the destruction complex consisting of axin, adenomatous polyposis coli (APC), and GSK3 β and the stabilization of β -catenin. Cytoplasmic β -catenin accumulates and is eventually imported into the nucleus, where it serves as a transcriptional coactivator of transcription factors of the TCF/LEF family.

The transcription factors of the LEF/TCF (lymphoid enhancer factor/T-cell-specific factor) family are the most downstream components of the Wnt signaling cascade. Today, four family members are known in mammals: LEF-1, TCF-1, TCF-3, and TCF-4. All share a homologous “HMG box” DNA-binding domain and recognize the conserved consensus sequence (5'-AGATCAAAGGG-3') in their target gene promoters, including Cyclin D1, MITE, c-myc, MMP-7, and others.

Wnt1 and Wnt3A are the predominant family members involved in melanocyte development and both activate β -catenin. β -catenin mutations were found in 6 out of 26 melanoma cell lines, but appeared to be rare among primary melanoma tumor specimens. Truncating mutations of the adenomatous polyposis coli (APC) gene that regulates β -catenin levels are also rare in melanoma. β -catenin is still required for melanoma survival even though mutations in Wnt signaling are rare (Takeda et al. 2000; Widlund et al. 2002).

5.12

Notch Signaling

The Notch signaling pathway is a key developmental cell–cell interaction mechanism, which regulates processes such as cell proliferation, cell fate, differentiation, or stem cell maintenance. All receptors and ligands are single-pass transmembrane proteins with large extracellular domains that consist primarily of epidermal growth factor (EGF)-like repeats. In mammals, four Notch receptors (Notch1–4) and five ligands (Jagged-1 and 2, and Delta-like [Dll] 1, 3, and 4) have been described (Greenwald 1998).

The interaction between ligand and Notch receptor results in two successive proteolytic cleavages. The first cleavage occurs extracellularly and is initiated by a metalloprotease of the ADAM family (TACE, tumor necrosis factor- α -converting enzyme). This allows the second cleavage to take place at the transmembrane domain, mediated by a protein complex with γ -secretase activity (presenilin, nicastrin, APH1, and PEN2) (reviewed in Fortini, 2002). The released intracellular domain of Notch (NIC) then translocates to the nucleus where it binds to CSL transcription factors (CBF1 in humans) and thereby activates transcription of Notch target genes via consensus Notch-binding site (5'-TGGGAA-3'). In the absence of Notch signaling, CSL functions as a transcriptional repressor via interactions with several corepressors.

The Notch signaling pathway is involved in tumorigenesis, as aberrant Notch signaling is frequently observed in certain cancers. Depending on the cell type and context, Notch can promote cell proliferation and cancer growth, or act as a tumor suppressor (Radtke and Raj 2003.; Wilson and Radtke 2006.). There is increasing evidence that Notch acts as an oncogene in the development of melanomas, with several receptors (Notch1 and Notch2), ligands (Jagged-1, Jagged-2, and Dll1) as well as target genes (HES1, HEY1, and MCAM) upregulated at early stages of melanocytes transformation and tumor progression (Hoek et al. 2004). Notch1 is thought to play an important role stage-specifically to promote the progression of primary melanoma (Balint et al. 2005).

5.13

NF- κ B (Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells)

NF- κ B proteins were initially identified as pivotal transcription factors in chronic inflammatory diseases (Sen and Baltimore 1986.). Accumulating evidence indicates that NF κ B is activated by a wide variety of stimuli such as cytokines and chemokines via

AKT-signaling, MAPK-signaling, or NIK (NF κ B interacting kinase) (Diaz-Meco et al. 1994; Kane et al. 1999; Ling et al. 1998; Ozes et al. 1999; Romashkova and Makarov 1999.; Sizemore et al. 1999). NF κ B is a homo- or heterodimeric complex consisting of proteins of the Rel-family. Rel-proteins can be divided into 2 groups depending on their mode of synthesis and transactivation properties. One class combines p65 (also known as REL A), REL B, and c-REL which are all directly synthesized in their mature forms. The second class consists of p105 (also known as NF κ B1) and p100 (also known as NF κ B2) which are synthesized as large precursors and matured via phosphorylation and proteolysis into p52 and p50, respectively. The most commonly detected dimers are p65/p50, p65/p65, and p50/p50. NF κ B recognizes the DNA consensus (5'-GGG(A/G)NN(C/T)(C/T)CC-3') (Amiri and Richmond 2005.).

In the absence of stimuli such as cytokines or chemokines, NF κ B binds with I κ B α protein and localizes in the cytoplasm. In the presence of stimuli, I κ B kinase (IKK) complex is activated via kinases including AKT, ERK, or NIK and then I κ B α is phosphorylated, ubiquitinated, and degraded. I κ B α degradation induces translocation of NF κ B from the cytoplasm to the nucleus, and target genes are transcriptionally activated.

Many NF κ B-regulated genes have been reported, which are related to carcinogenesis, including apoptosis (TRAF1, TRAF2, c-IAP1/2, ML-IAP, and survivin), proliferation (cyclin D1 and CDK2), metastasis (COX2, ICAM-1, VCAM-1, ELAM-1, and MMPs), and angiogenesis (CXCL-1/8, IL-1, and TNF).

Constitutive activation of NF- κ B is an emerging feature in melanoma and various target genes, CXCL1 and CXCL8, are highly expressed in most melanoma cell lines (Yang and Richmond 2001.). Furthermore, knockdown of IKKbeta reduces the growth of the melanoma lesions in mouse studies, and conditional deletion of Ikkbeta in melanocytes blocks HRAS-induced melanomagenesis. Taken together, NF κ B-signaling may play an important role in melanoma growth/survival and melanomagenesis (Yang et al. 2010).

5.14 SMAD/SKI

The transforming growth factor- β (TGF- β) family of growth factors comprises more than 40 members including TGF- β , Activins, Bone Morphogenetic proteins (BMP), and Nodal. They are ubiquitous multifunctional cytokines that regulate cellular activities, such as proliferation, differentiation, migration, survival, embryonic development, angiogenesis, or immune surveillance (Massagué 2000). Binding of the TGF family to their receptors causes the assembly of a receptor complex that phosphorylates Smad2/3, which in turn bind to Smad4 and accumulate in the nucleus where they act as transcription factors, usually via Smad-binding sites (5'-AGAC-3') (Nakao et al. 1997).

In normal tissues and cells, including melanocytes, transforming growth factor β (TGF- β) contributes to homeostasis by blocking cell-cycle progression via upregulation of the cyclin-dependent kinase inhibitors p15 and p21Waf-1, and downregulation of c-MYC (Reynisdóttir et al. 1995; Warner et al. 1999). There are other targets regulated by TGF- β -smad signaling, including VEGF, BMPR-II, ets2, and PAI-1.

The ski oncogene was discovered in its viral form, v-ski, as the transforming gene of the defective Sloan Kettering Virus (Stavnezer et al. 1981). SKI has been shown to interact with Smad2/3/4, recruit a transcriptional repressor of the N-CoR family and Histone deacetylases (HDACs), and then repress Smad-driven transcription (Khan et al. 2001).

Inactivation of the TGF- β pathway has been observed in a variety of human cancers including melanomas. Primary invasive melanomas *in vivo* exhibit nuclear and cytoplasmic localization of SKI, whereas in melanoma metastasis, SKI is mostly localized in the cytoplasm and expressed at very high levels (Reed et al. 2001). SKI might inhibit smad-driven transcription of p21 in melanoma, resulting in escaping TGF- β -induced anti-proliferative activity. SKI is also a potent stimulator of Wnt/ β -catenin signaling in human melanoma cells by binding to FHL2, which in turn activates MITF and Nr-CAM promoters in a β -catenin-dependent manner (Chen et al. 2003). SKI may participate as a regulator of melanoma progression by activating β -catenin signaling and repressing the TGF- β pathway.

5.15

STAT3 (Signal Transducer and Activator of Transcription 3)

STAT proteins were originally discovered as mediators of cytokine receptor signaling and are both cytoplasmic signaling molecules and nuclear transcription factors that activate diverse genes (Schindler et al. 1992; Shuai et al. 1992). There are seven STAT proteins (STAT1–4, 5A, 5B and 6) in mammals (Li 2008). In the canonical mode of JAK–STAT signaling, activation of the pathway is initiated by binding of a peptide ligand (e.g., a cytokine) to transmembrane receptors. This leads to receptor dimerization and cross-activation of receptor-associated JAK kinases (JAK1–3 or tyrosine kinase 2 (TYK2)), which in turn phosphorylate tyrosine residues in the cytoplasmic tail of the receptor. These phospho-tyrosine residues function as docking sites for latent cytoplasmic STAT proteins, which are then phosphorylated by JAK on a crucial C-terminal tyrosine residue near the 700 amino acid position. Phosphorylated STAT proteins dimerize via Src-homology 2 (SH2)-domain–phospho-tyrosine interactions and translocate to the nucleus, where they function as transcriptional activators, inducing expression of target genes via their consensus binding site (5'-TTC(N)nGAA-3').

STAT3 regulates genes related to survival/apoptosis (survivin, BCL2-XL, and MCL-1), proliferation (MYC and cyclin D1), and metastasis (HIF-1, VEGF, and MMP-2) though some of its targets are not yet known to be directly regulated by STAT3 (i.e., may be indirectly regulated downstream of STAT3).

Protein inhibitor of activated STAT3 (PIAS3) has been identified as an inhibitor of STAT3 and MITF and may also regulate melanocyte growth. Overexpression of PIAS3 in melanoma cell lines inhibits the transcriptional activity of both MITF and STAT3 and induces apoptosis (Levy et al. 2002).

The overexpression of activated Stat3 containing two cysteine residue mutations can mediate cellular transformation (Bromberg et al. 1999). Among the human cancers that display constitutively activated STAT3 are a variety of hematologic malignancies, including myeloma, leukemias, and lymphomas, as well as solid tumors, including breast, lung, prostate, ovarian cancers, and melanoma (Buettner et al. 2002). In melanoma, several receptor tyrosine kinases including c-Met, EGFR, ERBB2, and ERBB4, which are known to activate src kinases, are overexpressed or activated. STAT3 inhibition in melanoma cell lines or melanoma tumor models has been shown to induce cell death/tumor regression (Niu et al. 1999, 2001), inhibit angiogenesis (Xu et al. 2005), prevent metastasis (Xie et al. 2004), and activate antitumor immune responses (Wang et al. 2004).

5.16

HIF1A (Hypoxia-Inducible Factor 1 α)

HIF-1 is the key transcriptional regulator of the cellular response to a hypoxic environment. HIF1 plays a key role in many cellular processes that participate in responses to reduced oxygen and energy supply. HIF1 consists of a heterodimer with HIF1 α and HIF1 β , and binds to the hypoxia responsive element (HRE, 5'-RCGTG-3'). Under normoxic conditions, HIF-1 α is hydroxylated on proline residue 402 (Pro-402) and/or Pro-564 by prolyl hydroxylase domain protein 2 (PHD2). Prolyl-hydroxylated HIF-1 α is bound by the von Hippel–Lindau tumor suppressor protein (VHL), which recruits an E3-ubiquitin ligase that targets HIF-1 α for proteasomal degradation (Maxwell et al. 1999). Under hypoxic conditions, the prolyl and asparaginyl hydroxylation reactions are inhibited by substrate (O₂) deprivation and/or the mitochondrial generation of reactive oxygen species (ROS), which may oxidize Fe(II) present in the catalytic center of the hydroxylases.

HIF1 target genes have been identified in tumors, and are related to angiogenesis (VEGF), motility (CXCR4), proliferation (BNIP3), and the reprogramming of cancer metabolism (SLC2A1, SLC2A3, LDHA, MCT4, and PDK1).

HIF1 α expression is regulated at the transcriptional and translational levels by the PI3K/AKT and MAPK/ERK pathways. Interestingly, PTEN loss or BRAF^{V600E}, which overactivate the PI3K/AKT or MAPK/ERK pathways, respectively, increase HIF1 α expression and melanoma survival under hypoxic condition through HIF-1 α (Kumar et al. 2007). HIF1 α has also been reported to be an MITF target gene (Buscà et al. 2005).

5.17

Tbx-2/3 (T-Box Binding Protein 2/3)

Members of the T-box family of transcription factors play important roles in the regulation of cell-fate decisions and morphogenesis during development. This family binds the 20 nucleotide partially palindromic sequence T[G/C]ACACCTAGGTGTGAAATT. Brachyury can bind as a monomer or a dimer whereas Tbx-2 binds as a monomer to Brachyury single half-sites, recognizing the consensus sequences GTGTGA, GGGTGA, or GTGTTA (Carreira et al. 1998). Several T-box genes are involved in the progression of cancer. Amplification of Tbx2/3 is reported in breast cancer (Sinclair et al. 2002; Fan et al. 2004). Both are also expressed in normal melanocytes and have been found to be strongly upregulated in a subset of melanoma cell lines (Carreira et al. 1998, Hoek et al. 2004; Vance et al. 2005).

Tbx2 and Tbx3 function as transcriptional repressors. Tbx2 represses TYRP-1, which is one of the pigmentation enzymes, p21, and p19. Through its targeting of p21 and p19, Tbx2 may be required to maintain proliferation and suppress senescence in melanomas (Vance et al. 2005). E-cadherin is also known to be a target gene of Tbx3, and potentially Tbx2. Tbx3 may be related to the transition from radial growth phase (RGP) to vertical growth phase (VGP) and could contribute to metastatic potential via suppression of E-cadherin expression.

TBX2 is regulated transcriptionally and post-translationally by the p38 stress signaling pathway in response to UVC irradiation (Abrahams et al. 2008). TBX2 is also reported to be an MITF target gene (Carreira et al. 2000).

5.18

C-MYC

c-Myc is a basic Helix-Loop-Helix Leucine Zipper protein, which binds to a subset of E-box (5'-CANNTG-3') consensus sequences. This gene was discovered as a translocated oncogene in Burkitt's lymphomas, resulting in c-MYC overexpression under the control of the immunoglobulin enhancer or promoter (Taub et al. 1982). Dysregulated expression and/or amplification of C-MYC have been known to be an important event for many tumors. The c-myc locus (8q24) is amplified in 30–50% melanoma (Kraehn et al. 2001). Moreover, the BRAF^{V600E} and NRAS(Q61R)-specific senescence program is suppressed by c-myc overexpression in melanoma (Zhuang et al. 2008).

c-MYC-dependent transactivation requires heterodimerization with a partner protein Max. This dimerization with Max is also essential for Myc proliferative and oncogenic function (Amati et al. 1993; Ferré-D'Amaré et al. 1993.). The inhibition of the dimerization with Max might be a potential therapeutic target. Thus far, the most “drugable” transcription factors are ligand-dependent ones (particularly nuclear hormone receptors), although efforts to successfully target other transcription factors will hopefully bear fruit in the future.

5.19

p53

p53 is one of the most important human tumor suppressors (Lozano 2007; Riley et al. 2008). It plays important roles in controlling the DNA damage response, cell-cycle progression (p21, p16, p14, etc.), and apoptosis (APAF-1, PUMA, etc.) by regulating its targets transcriptionally. Deletion or loss-of-function mutations are found in diverse human cancers (see also Chap. 4).

UV causes DNA damage in human skin and correlates with melanoma incidence. Pigmentation may prevent epidermal cells from UV-induced DNA damage. p53 plays a role in the pigmentation response via transcriptional activation of the POMC promoter in keratinocytes, which encodes α -melanocyte stimulating hormone (Cui et al. 2007).

A number of groups have reported mutational analysis of the *p53* gene in melanoma. Low frequency (0 – 10%) *p53* mutation or loss of heterozygosity in melanoma has been observed (Volkenandt et al. 1991; Castresana et al. 1993; Lübbe et al. 1994; Montano et al. 1994; Sparrow et al. 1995; Hartmann et al. 1996; Papp et al. 1996), suggesting that there are additional mechanisms to suppress the function of p53. One mechanism involves the MDM2 gene, which encodes an E3 ubiquitin ligase that binds directly to p53 and ubiquitinates it, targeting p53 for proteosomal degradation. In melanoma progression, MDM2 has been shown to be highly expressed in 6% of dysplastic nevi, 27% of melanoma in situ, and 56% of invasive primary and metastatic melanomas (Polsky et al. 2001).

5.20

Conclusions

The importance of transcription factors in melanocyte development and melanomagenesis has been summarized in this chapter (Table 5.1). Multiple transcription factors have been shown to be activators or suppressors of melanoma proliferation, survival, metastasis, and apoptosis. The complexity of their activities remains to be fully elucidated, and will undoubtedly employ newer technologies including analyses of epigenetic marks, chromatin remodeling, and identification of coactivator or corepressor multiprotein complexes. For most transcription factors we do not currently have effective therapeutic strategies available to permit their pharmacologic modulation. Exceptions include nuclear hormone receptors, which require small molecule ligand-dependent activation or certain drugable strategies affecting pathways that indirectly control transcription factors. Hopefully increasing accumulation of information about transcription factor biochemistry and the pathways which modulate their activities will provide new clues to novel therapeutic targeting strategies aimed at transcription factors in melanoma.

Table 5.1 Overview of relevant transcription factors for melanoma

Gene	Binding sequences	Cancer-related biological function	Cancer-related target genes
MITF	CA[C/T]GTG	Cell-cycle progression, survival	CDK2, p16, p21, TBX2, TRPM1, HIF1A, BCL2, c-MET
CREB	TGANNTCA	Tumorigenicity	CCND1, MITF (by EWS-ATF1)
SOX10	[A/T][A/T]CAA[A/T]G	Unknown	MITF
PAX3	GT[T/C][C/A][C/T][G/C][G/C], TAAT	Motility (by PAX3-FOXO1)	MITF, PTEN, BCL-XL, c-MET
Snaill superfamily	CAGGTG	Epithelial–mesenchymal transition	E-cadherin (CDH1), claudin, occludin
FOXD3	A[A/T]T[A/G]TTTGTTT	Suppressor of growth	MITF
ETS family	GGA[A/T]	Tumorigenicity	MITF (not direct target)
BRN2	ATGC, AAAT	Metastasis	MITF
AP-1	TGA[G/C]TCA	Proliferation, metastasis	ATM, XPA, cyclin A, CCND1, MMP2, TNF- α
AP-2	GCCNNNGGC	Melanoma progression	p21, c-KIT, BCL2, MMP2, CDH1
LEF/TCF/ β -catenin	AGATCAAAGGG	Survival	CCND1, MITF, c-MYC, MMP7
Notch (NIC)	TGGGAA	Melanoma progression	HES, HEY1, MCAM
NFkB	GGG[A/G]NN[C/T][C/T]CC	Growth, survival	c-IAP1/2, survivin, CCND1, CDK2, CXCL-1/8
SMAD/SKI	AGAC	Melanoma progression	p21, VEGF, ETS2, PAI-1
STAT3	TTCNnGAA	Melanoma survival, angiogenesis	Survivin, BCL-XL, MCL-1, HIF1A, c-MYC, CCND1, MMP2, VEGF

HIF1A	[A/G]CGTG	Angiogenesis, metastasis, proliferation	VEGF, CXCR4, BNIP
TBX2/3	G[G/T]GTGA, GTGTTA	Metastasis	p21, p19, CDH1
c-MYC	CANNTG	Suppress the senescence	BCL2
p53	[A/G] [A/G] [A/G]C[A/T] [T/A]-G[C/T] [C/T] [C/T]	Cell-cycle progression, DNA damage response	p21, p16, p14, PUMA

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Abstract MicroRNAs (abbreviated: miRNAs) represent a class of small noncoding RNAs, first described in the nematode *Caenorhabditis elegans*. In 1993, the labs of Victor Ambros (Lee et al. 1993) and Gary Ruvkun (Wightman et al. 1993) discovered lin-4, the first member of the inexorably growing miRNA family of molecules, in close cooperation. Interestingly, it was not until the year 2000 that Reinhart and colleagues detected a second miRNA species – let-7 (Reinhart et al. 2000). The finding that the sequence of let-7 was conserved in a large variety of Metazoens from *Drosophila* to humans [in contrast to lin-4 which is exclusively expressed in *Caenorhabditis*; (Pasquinelli et al. 2000; Slack et al. 2000)] fueled miRNA research and revealed that this class of molecules is involved in the regulation of gene expression at a posttranscriptional level in presumably every multicellular organism.

To date, almost 800 distinct miRNA species have been identified in the human genome [<http://microrna.sanger.ac.uk/>; (Griffiths-Jones et al. 2008)]. Those are estimated to regulate the expression of as much as 30% of all human transcripts (Lewis et al. 2005). MiRNAs are demonstrably involved in the physiological regulation of multiple cellular processes, such as proliferation, apoptosis, cell-cycle regulation, and differentiation (John et al. 2004; Johnson et al. 2005; Kiriakidou et al. 2004; Krek et al. 2005; Lee et al. 2005; Lim et al. 2005). As a consequence, abnormalities in miRNA activity were found to contribute to the pathogenesis and progression of various types of human cancers [reviewed in (Mirnezami et al. 2009; Visone and Croce 2009)], including malignant melanoma [reviewed in (Mueller and Bosserhoff 2009)].

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6.1 miRNA Biogenesis and Function

6.1.1 Transcription of miRNA Genes and Processing in the Nucleus

MicroRNA genes are embedded in intergenic as well as intragenic regions of the human genome, either encoding a single miRNA species or a cluster of multiple miRNAs in a polycistronic manner (Lee et al. 2002). In the first step of miRNA expression, miRNA genes are transcribed into pri-miRNAs (primary miRNA transcripts; Fig. 6.1). In most cases, transcription is accomplished by RNA polymerase II, resulting in 5'-methyl-guanosine capped and polyadenylated pri-miRNAs which contain local stem-loop structures and are up to

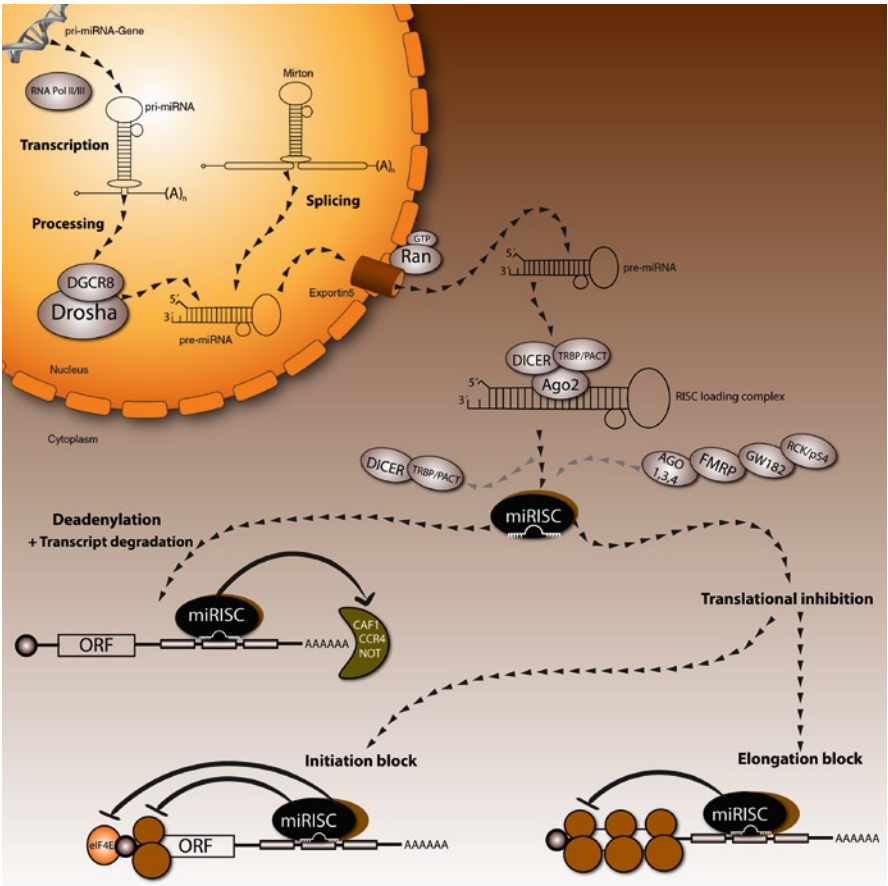


Fig. 6.1 Schematic overview of miRNA biogenesis and function. Please refer to Sect. 6.1 for detailed explanations on the molecular mechanisms involved in each step

several kilo bases in length (Cai et al. 2004; Lee et al. 2004). Some specific miRNAs are initially transcribed by RNA polymerase III (Borchert et al. 2006). While still in the nucleus, the pri-miRNA is endonucleolytically cleaved by the so-called microprocessor complex composed of the RNase III enzyme Drosha (RNASEN) and its co-factor DGCR8 [DiGeorge syndrome critical region on chromosome 8; also known as Pasha (Partner of Drosha) in *D. melanogaster* and *C. elegans*] (Landthaler et al. 2004; Lee et al. 2003). DGCR8 interacts with the ~33bp stem-loop as well as with the adjacent unpaired flanking regions within the pri-miRNA, thus supporting Drosha-mediated cleavage in the stem region, about 11bp away from the junction of ssRNA (single-stranded RNA) to dsRNA (double-stranded RNA) (Han et al. 2006; Zeng and Cullen 2005). The resulting pre-miRNA (~70bp in length) is rapidly translocated to the cytoplasm via the Ran-GTP-dependent nuclear export factor exportin 5 (EXP5), a member of the nuclear transport receptor family (Kim 2004). Export of only correctly processed pre-miRNAs is ensured by recognition of the >14bp dsRNA stem together with a short (1–8 nucleotides) 3' overhang (Zeng and Cullen 2003). Interestingly, a number of pre-miRNAs can be exported from the nucleus without undergoing processing by Drosha. These so-called mirtrons are located in very short introns and are capable of forming a hairpin resembling pre-miRNA molecules after they were released from their host transcripts by splicing and debranching (Berezikov et al. 2007).

6.1.2

Cytoplasmic Processing and Modes of miRNA Mediated Gene Silencing

In the cytoplasm, maturation of pre-miRNAs occurs via a multi-enzyme complex called RISC loading complex (RLC). This RLC is composed of the RNase III enzyme Dicer, the double-stranded RNA-binding domain proteins TRBP (TAR RNA-binding protein) and PACT (protein activator of PKR), as well as AGO2 (Argonaute-2), which builds the core of the complex (Gregory et al. 2005; MacRae et al. 2008). TRBP and PACT facilitate Dicer-mediated cleavage of the pre-miRNA, which occurs near the terminal loop and results in a RNA duplex of ~22 nucleotides with two nucleotide overhangs on each 3' terminus (Hutvagner et al. 2001; Knight and Bass 2001). In some cases, AGO2 – which exhibits robust RNaseH-like endonucleolytic activity – can support Dicer processing by cleaving the 3' arm of specific pre-miRs (Diederichs and Haber 2007). Subsequently, Dicer and its interactors TRBP and PACT dissociate from the complex, and the miRNA duplex is separated into the guide strand (which is complementary to the target mRNAs and is thus functional in gene silencing) and the passenger strand (miRNA*) which usually gets degraded. It would appear that there is no universal helicase responsible for the unwinding of the miRNA duplex, but specific helicases may regulate subgroups of miRNAs differentially (Winter et al. 2009). In other cases, a helicase is not required at all for duplex unwinding (Pillai et al. 2005; Wu and Belasco 2005). The guide strand is characterized by the presence of a thermodynamically less stable base pair at the 5' end of the duplex and is loaded onto the RISC (RNA induced silencing complex) after unwinding (Khvorova et al. 2003). Of note, miRNA* strands are not always simply by-products of miRNA biogenesis, but are sometimes also acting as functional miRNAs on the miRISC (Chiang et al. 2010; Ghildiyal et al. 2010; Okamura et al. 2009).

The assembly of the miRISC, also called miRNP (micro-ribonucleoprotein), is a dynamic process coupled with the preceding steps of pre-miRNA processing. Key components of the miRISC are proteins of the Argonaute (AGO) family, FMRP (fragile X mental retardation protein), and P-body components including GW182 and RCK/p54, which are essential for inducing miRNA-mediated gene repression (Filipowicz et al. 2008). Guided by the mature miRNA, the miRISC subsequently binds to target sequences in the 3' untranslated regions (3'UTRs) of regulated transcripts, in order to inhibit their translation into functional protein. To date, the general rules for the initial miRNA:mRNA interaction, which are fundamental for target recognition, are only incompletely determined experimentally and bioinformatically (Brennecke et al. 2005; Doench and Sharp 2004; Grimson et al. 2007; Lewis et al. 2005; Nielsen et al. 2007). In animals, miRNAs almost exclusively bind to their target mRNAs with imperfect complementarity. Nevertheless, an indispensable prerequisite for efficient transcript targeting is continuous base-pairing of miRNA nucleotides 2–8, which are called the miRNAs “seed-sequence” (Bartel 2009). It actually is this miRNA seed which is the major determinant of a miRNAs target repertoire.

The resulting miRNA-induced posttranscriptional silencing of target genes is mediated either by destabilization of the corresponding mRNA (Behm-Ansmant et al. 2006b; Giraldez et al. 2006; Wu et al. 2006; Wu and Belasco 2005) or by repression of protein translation (Pillai et al. 2005; Standart and Jackson 2007); both pathways acting cooperatively but yet independently of each other. Current knowledge suggests that destabilization of target mRNAs starts with recruitment of the P-body component GW182 (glycine-tryptophan protein of 182 kDa) by Argonaute proteins (Till et al. 2007). GW182 subsequently mediates binding of the CAF1:CCR4:NOT1 deadenylase complex to the target mRNA. Deadenylation is then followed by removal of the 5'-methyl-guanosine cap via the DCP1:DCP2 decapping complex ultimately leading to 5'→3' exonucleolytic degradation of mRNA by exonuclease XRN1 (Behm-Ansmant et al. 2006a; Eulalio et al. 2007, 2009). By contrast, there still is a lack of consensus concerning the mechanism(s) by which miRNAs induce repression of translation. While many experiments refer to the initiation of translation as a target for repression, there is also evidence that various post-initiation steps could be affected [reviewed in (Chekulaeva and Filipowicz 2009)]. Present research aims to unravel whether miRNAs are actually capable of controlling translation by multiple mechanisms or if these discrepancies were due to different experimental approaches utilized in the past (Cannell et al. 2008; Kong et al. 2008). Figure 6.1 summarizes the mechanisms involved in miRNA biogenesis and function.

6.1.3

Regulation of miRNA Biogenesis, Function and Decay

The general pathway of miRNA biogenesis and function – as well as the clearance of miRNAs through decay – is complicated by a large number of regulatory mechanisms, in which a vast quantity of yet unidentified proteins is likely involved. There may be specific alterations to the pathway for every individual miRNA or at least distinct subgroups of miRNAs [reviewed in (Krol et al. 2010)].

The expression of miRNAs can be regulated at the level of transcription [reviewed in (Davis and Hata 2009; Turner and Slack 2009)] or at various steps during processing of the

pri-miR into mature miRNA [reviewed in (Davis and Hata 2009)]. The latter mechanism commonly involves regulation of constituents of the miRNA processing machinery, like Drosha/DGCR8 (Han et al. 2009; Triboulet et al. 2009) and Dicer/TRBP (Chendrimada et al. 2005; Melo et al. 2009; Paroo et al. 2009). Additionally, accessory proteins can directly interact with miRNA precursors either repressing or stimulating their further processing [reviewed in (Winter et al. 2009)]. In contrast, modulation of miRNA function is mainly based on mechanisms affecting proteins of the miRISC, predominantly AGO2 and GW182 [reviewed in (Krol et al. 2010)]. With regards to melanoma, it very recently has been implicated that transcriptional regulation of Dicer by MITF might be a major determinant in melanocyte cell survival and differentiation [(Levy et al. 2010); please refer to Sect. 6.2 for detailed information].

To date, little is known about the regulation of miRNA decay. Nevertheless, recent findings implicate that sequences present at the 3' end of specific miRNAs or, more generally, enzyme catalyzed modifications to the 3' end of miRNAs may determine the rate of miRNA clearance (Hwang et al. 2007; Jones et al. 2009; Katoh et al. 2009). Interestingly, degradation of mature miRNAs seems to depend on their activity: In the absence of its complementary targets, a miRNA may be specifically released from the miRISC. Thereby, its 5' end gets accessible to the 5' → 3' exonuclease XRN-2, which subsequently degrades the miRNA (Chatterjee and Grosshans 2009).

These findings clearly show that miRNAs are not only active regulators of gene expression but are themselves subject to sophisticated control. Adding even more complexity, miRNAs have recently been shown to not exclusively repress gene expression but to conversely enhance translation of specific transcripts under certain cellular conditions – although these cases are clearly the exception rather than the rule (Vasudevan et al. 2007, 2008).

6.2

Impact of Specific miRNAs on Melanomagenesis

In contrast to other types of tumors, studies on the impact of specific miRNA species on melanomagenesis had not been conducted before the year 2008. Nevertheless, several miRNAs deregulated in melanoma cells compared to normal melanocytes have been characterized in regards to their target genes as well as their impact on melanoma cell function since then (Fig. 6.2 and Table 6.1). The following paragraph aims to integrate those findings in their meaningful context and to point out how miRNA-related research expanded our knowledge on the molecular mechanisms contributing to formation and progression of malignant melanoma.

6.2.1

Networking of miRNAs and MITF

It is not surprising that the very first miRNA identified to influence melanoma progression is involved in modulating expression levels of the key regulator of melanocyte cell fate – microphthalmia-associated transcription factor (MITF; see Chaps. 5 and 12).

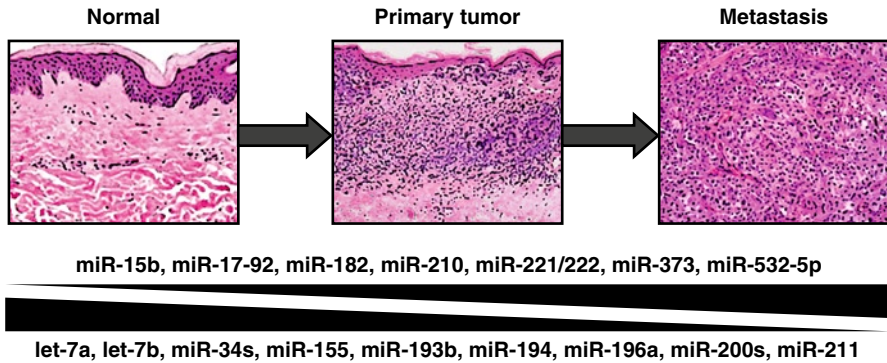


Fig. 6.2 Schematic overview of miRNAs deregulated during melanoma formation and progression [Reprinted from (Mueller and Bosserhoff 2009), modified. With kind permission from the *British Journal of Cancer*]

In their study, Bemis et al. (2008) set out to characterize an allele for melanoma susceptibility previously determined to be located on chromosome 1p22 (Gillanders et al. 2003; Walker et al. 2004). It turned out that this genomic locus encodes a miRNA – miR-137 – capable of negatively regulating MITF expression (Bemis et al. 2008). Interestingly, the miR-137 primary transcript harbors a significantly higher number of a 15bp VNTR (variable number of tandem repeats) in a subset of melanoma cell lines displaying high MITF expression than it does in melanoma cell lines displaying low levels of MITF. Those extra copies of the 15bp VNTR alter the pri-miR-137 secondary structure in a way precluding its further processing into mature miR-137 (Bemis et al. 2008). Strikingly, re-expression of functionally active miR-137 in those cell lines resulted in a decline of MITF levels.

As already suggested in the report cited above, it meanwhile turned out that several other miRNAs are involved in regulating MITF as well. Also based on a melanoma associated genomic aberration – amplification of chromosomal locus 7q31-34, which harbors the *c-MET* and *BRAF* oncogenes – Segura et al. (2009) detected enhanced expression of miR-182 in melanoma cell lines and tissue samples. miR-182 levels increased with tumor progression and were additionally inversely correlated with the expression of MITF and FOXO3 (a transcription factor of the forkhead family). Both transcription factors were confirmed to be direct targets for miR-182. It was, furthermore, shown that enforced expression of this miRNA stimulates the migration of melanoma cells *in vitro* as well as their metastatic potential *in vivo*, whereas silencing of miR-182 impedes melanoma cell invasion and triggers apoptosis (Segura et al. 2009). The stimulatory effect of miR-182 over-expression on melanoma cell migration was abolished by concomitant over-expression of MITF or FOXO3 making those proteins the key mediators of miR-182's effects.

Interestingly, it has been reported that cancer cells can circumvent miRNA mediated regulation of oncogenes which they are addicted to by expressing transcripts with shortened 3'UTRs (Mayr and Bartel 2009). Thereby, selective pressure favors cells which generate alternative transcripts of an oncogene missing the miRNA target sequence(s) in their 3'UTR. Prompted by those findings, Goswami et al. (2010) unraveled that a specific MITF isoform harboring a shortened 3'UTR is preferentially expressed by their set of melanoma

Table 6.1 Summary of expression profiling as well as functional data available on miRNAs deregulated in melanoma cells. Question marks in brackets indicate lack of compelling evidence. Due to space limitations only most prominent miRNAs were included. Please refer to [Sects. 6.2](#) and [6.3](#) for details

miRNA	Gain/loss in melanoma cells	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)
<i>let-7a</i>	↓	Microarray, qRT-PCR, northern blot, functional tests	Not yet determined	N-Ras, integrin beta3	Muller and Bosserhoff (2008); Ma et al. (2009); Chen et al. (2010); Philippidou et al. (2010)
<i>let-7b</i>	↓	Microarray, qRT-PCR, functional tests	Not yet determined	Cyclin D1	Schultz et al. (2008); Chen et al. (2010)
<i>miR-15b</i>	↑	qRT-PCR, functional tests	Not yet determined	None	Satzger et al. (2010)
<i>miR-17-92</i>	↑	Microarray, qRT-PCR	Not yet determined	BIM(?)	Mueller et al. (2009); Levati et al. (2009); Chen et al. (2010); Philippidou et al. (2010)
<i>miR-34a</i>	↓	qRT-PCR, northern blot	Hypermethylation	None	Lodygin et al. (2008)
<i>miR-34b/c</i>	↓	qRT-PCR, functional tests	Hypermethylation	MET(?)	Lujambio et al. (2008); Migliore et al. (2008)
<i>miR-137</i>	↓(?)	qRT-PCR, functional tests	Amplification of a VNTR in the 5'UTR of the pri-miRNA	MITF	Bemis et al. (2008)
<i>miR-182</i>	↑	qRT-PCR, functional tests	Amplification of 7q31-34	MITF, FOXO3	Segura et al. (2009)

(continued)

Table 6.1 (continued)

miRNA	Gain/loss in melanoma cells	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)
<i>miR-193b</i>	↓	Microarray, qRT-PCR, functional tests	Not yet determined	Cyclin D1	Chen et al. (2010)
<i>miR-196a</i>	↓	Microarray, qRT-PCR, functional tests	Not yet determined	HOX-B7, HOX-C8	Braig et al. (2010); Mueller and Bosserhoff (2010a); Philippidou et al. (2010); Caramuta et al. (2010)
<i>miR-210</i>	↑	qRT-PCR	Hypoxia or constitutive HIF-1α activity (?)	MNT(?)	Zhang et al. (2009); Satzger et al. (2010)
<i>miR-221/222</i>	↑	Microarray, qRT-PCR, functional tests	Loss of PLZF expression, MITF level, down-regulation of hPNP ^{old-35}	c-KIT, p27 ^{Kip1}	Felicetti et al. (2008); Igoucheva and Alexeev (2009); Das et al. (2010); Mueller et al. (2009); Philippidou et al. (2010)
<i>miR-532-5p</i>	↑	qRT-PCR, functional tests	Not yet determined	RUNX3(?)	Kitago et al. (2009)

cell lines in contrast to normal melanocytes. This alternative MITF transcript lacks the miR-137 and miR-182 binding sites described before but yet contains two target sequences for another miRNA, miR-340 (Goswami et al. 2010). The authors were able to show that miR-340 actually inhibits expression of the alternative MITF transcript in their melanoma cells. Even more remarkable, a protein commonly over-expressed in melanoma and termed CRD-BP [coding region determinant binding protein; (Elcheva et al. 2008)] is able to bind to the alternative MITF 3'UTR, thereby masking the miR-340 target sites and restoring MITF expression (Goswami et al. 2010). On a functional level, either inhibition of miR-340 or over-expression of CRD-BP resulted in a significantly enhanced ability of melanoma cells to form colonies in soft agar.

In addition to these findings, Haflidadottir et al. (2010) attempted to perform a comprehensive analysis on miRNA binding sites located in the MITF full length 3'UTR. Although not paying attention to a potential alternative 3'UTR shortening, they identified miR-148 and miR-101 additional regulators of MITF expression in melanoma cell lines (Haflidadottir et al. 2010).

Taking all these findings into consideration, what is the impact of miRNA mediated regulation on MITF expression in melanomagenesis? Reports on the role of MITF in formation and progression of malignant melanoma had initially been controversial. Nowadays a model is proposed in which an intermediate level of MITF expression favors the proliferation and tumorigenic potential of melanoma cells, while a level too high results in cell cycle arrest and differentiation and a level too low results in cell cycle arrest and apoptosis (see Chaps. 4, 5 and references therein). Although expression of MITF has already been shown to be regulated through a plethora of mechanisms (including e.g., transcriptional control and post-translational modifications), miRNAs as well as 3'UTR binding proteins (acting as “target protectors”) seem to represent an additional layer of complexity in adjusting MITF levels. Most likely, in melanoma cells the favored outcome “intermediate MITF level” can be achieved by deregulation of every single wire (or a combination of several wires) in the MITF network. Hereby, deregulation of miRNA-based mechanisms is one way to adjust MITF at the preferred level or to fine tune it, respectively. This may also provide additional insights into melanomas heterogeneity regarding MITF expression.

However, MITF is not only subject to regulation by miRNA – vice versa MITF regulates the expression of a subset of miRNAs in melanoma cell lines and may even influence global miRNA expression in cells of the melanocytic lineage. Oszolak et al. (2008) identified the putative transcription start sites of as much as 175 miRNAs by combining nucleosome positioning patterns with chromatin immunoprecipitation (ChIP)-chip screens for promoter signatures. This report significantly contributed to our current knowledge on the characteristics of miRNA promoters. Furthermore, the authors utilized the data obtained to identify a subset of 10 miRNAs and miRNA clusters, respectively, whose expression is MITF regulated by screening *miRNA* promoters for E-box elements (the binding sites for MITF in gene promoters; see Chap. 5). The cohort of miRNAs identified to be MITF transcriptional targets includes e.g., miR-148b, miR-221/222, the miR-106a-363 cluster, miR-125, as well as several members of the let-7 family (Oszolak et al. 2008). Interestingly, some of these miRNAs have already been proven to target genes playing a role in pigmentation and survival of melanocytes or to be involved in melanomagenesis.

Additionally, Levy et al. (2010) recently reported that MITF is able to transcriptionally regulate Dicer expression in melanocytes. Their study started with the observation that during melanocyte differentiation a population of miRNAs was up-regulated at the pre-miR level, whereas another population of miRNAs displayed stabile pre-miR but yet enhanced mature miRNA levels. Subsequently, it turned out that MITF is capable of inducing Dicer transcription by interacting with two E-box elements in the *Dicer* promoter thus enforcing pre-miRNA processing (Levy et al. 2010). Interestingly, melanocyte specific knock-out of Dicer in an *in vivo* mouse model resulted in a profound loss of both melanocyte stem cells and differentiated melanocytes. The authors assigned this impressive impact of Dicer on melanocyte survival to its ability to enhance expression of the miR-17-92 cluster miRNAs. Hereby, miR-17 targeting of the pro-apoptotic Bim (Bcl-2 interacting mediator of cell death) protein may play a major role (Levy et al. 2010) and further links MITF-mediated regulation of Dicer to mechanisms involved in melanomagenesis.

These findings indicate that the melanocyte specific transcription factor MITF might exert its powerful impact on pigmentation and lineage survival genes not only by direct transcriptional mechanisms but that its effects are further enhanced – or even potentiated – by miRNAs as intermediates. Obviously, this will also enhance – or even potentiate – its oncogenic properties in melanoma formation and progression.

6.2.2

Oncogenic Role of miR-221/222 in Melanoma

Clustered on the X-chromosome, miR-221 and miR-222 are thought to be transcribed as a common precursor and to regulate overlapping or functionally related targets. Reports on an over-expression of these miRNAs in several types of tumors in which they regulated expression of the c-KIT receptor tyrosine kinase [e.g., (Felli et al. 2005; He et al. 2005; le Sage et al. 2007)] led Felicetti et al. (2008) to investigate a potential involvement of miR-221/222 in melanomagenesis (for further information on c-KIT in melanoma please refer to Chap. 7). They unraveled that loss of the tumor suppressive protein promyelocytic leukemia zinc finger (PLZF) leads to a stepwise up-regulation of miR-221/222 during melanoma progression (Felicetti et al. 2008). These two miRNAs in turn are able to induce two distinct but functionally convergent oncogenic pathways by repressing c-KIT as well as p27^{Kip1} (CDKN1B). In accordance, over-expression of miR-221/222 in a moderately aggressive melanoma cell line resulted in an enhancement of proliferation rate, migratory and invasive potential, as well as anchorage-independent growth, paralleled by a significantly increased *in vivo* tumorigenicity (Felicetti et al. 2008). Vice versa, treatment of highly aggressive metastatic melanoma cells with inhibitory molecules (antagomiRs) against miR-221/222 not only caused obverse effects *in vitro*, but – most strikingly – effectively inhibited *in vivo* tumor growth after bolus intratumor injection.

Expanding those findings, Igoucheva and Alexeev (2009) implicated that loss of c-KIT receptor expression in melanoma might be completely depending on miR-221/222 over-expression instead of being related to down-regulation of the transcription factor AP-2 (see Chap. 5) as previously reported. This conclusion was drawn as there was no correlation detectable between AP-2 and c-KIT expression levels in their set of 27 melanoma cell lines

but a strong inverse correlation of c-KIT and miR-221 expression levels was observed (Igoucheva and Alexeev 2009).

Including data generated in other tumor types, Howell et al. (2010) constructed an interesting network focusing miR-221/222's regulatory effects in melanoma (Howell et al. 2010). It has been demonstrated that miR-221/222 regulate expression of PTEN in aggressive non-small lung cancer and hepatocarcinoma cells (Garofalo et al. 2009). In the light of an increasingly important role of the AKT/PI3K pathway in melanomagenesis as well as a confusingly low rate of PTEN mutations detected in melanoma tissue samples (see Chap. 7), studies investigating a miRNA-mediated regulation of PTEN seem to be a promising option.

Additionally, Das et al. (2010) reported the existence of a 3' → 5' exonuclease, human polynucleotide phosphorylase (hPNPase^{old-35}), specifically degrading mature miR-221 in melanoma cells. In response to over-expression of hPNPase^{old-35}, the cellular miR-221 level decreased and its repressive impact on p27^{Kip1} expression was abolished (Das et al. 2010). As hPNPase^{old-35} is a type I interferon (IFN) inducible protein, the authors were able to unravel a pathway in which IFN-β mediated growth arrest is based on induction of hPNPase^{old-35} which subsequently degrades miR-221 and therefore triggers up-regulation of p27^{Kip1}. Of note, hPNPase^{old-35} had previously been shown to degrade c-Myc mRNA thereby causing G₁ cell cycle arrest by p27^{Kip1} activation involving another pathway (Sarkar et al. 2003, 2006). Nevertheless, over-expression of miR-221 in melanoma cells was sufficient to render them resistant against IFN-β mediated growth arrest (Das et al. 2010).

In conclusion, miR-221/222 have already been established to be potent oncogenic miRNAs in melanoma. Several mechanisms are involved in regulating their expression, including transcriptional control by PLZF (Felicetti et al. 2008) and MITF (Ozsolak et al. 2008), as well as positive and negative feedback loops involving their target genes [reviewed in (Howell et al. 2010)] and specific exonucleolytic degradation by hPNPase^{old-35} (Das et al. 2010). Although up-regulation of miR-221/222 may not be the sole cause for loss of c-KIT in melanoma cells, miRNA-mediated c-KIT repression could serve as an additional mechanism to escape c-KIT dependent apoptosis in a subset of melanomas harboring activating mutations in BRAF (Igoucheva and Alexeev 2009). Further, miR-221/222 over-expression may be responsible for resistance against IFN-β treatment (Becker et al. 2002) in at least some melanomas (Das et al. 2010).

6.2.3

Tumor-Suppressive Role of let-7 miRNA Family Members in Melanoma

One of the major breakthroughs regarding miRNAs in biomedical research took place in 2005 when Frank Slack's group reported a causal link between down-regulation of miRNA let-7 and lung cancer formation (Johnson et al. 2005). Thereby, the tumor-suppressive effect of let-7 miRNA family members in lung carcinomas is based on their potential to regulate expression of the RAS oncogene. Strikingly, it meanwhile turned out that loss of the expression of at least some let-7 family members seems to be a common event in the progression of several types of human cancers [reviewed in (Boyerinas et al. 2010)] – and that this is true for melanoma as well.

Performing an expression pattern analysis on 157 different miRNAs in laser-microdissected tissues comprising ten benign melanocytic nevi and ten primary melanomas, Schultz et al. (2008) found five members of the let-7 family (let-7a, let-7b, let-7d, let-7e, and let-7g) to be strongly down-regulated in the melanoma samples. Focusing only let-7b in their subsequent experiments, they showed that introducing artificial let-7b molecules into melanoma cells decreased levels of a set of cell cycle regulators, in detail cyclins D1, D3 and A as well as cyclin-dependent kinase (CDK) 4 (Schultz et al. 2008). While the authors were able to give some evidence for a direct interaction of let-7b with the cyclin D1 3'UTR, they also point out that at least a few of the remaining let-7b effects described might be rather indirect. Nevertheless, transfection of melanoma cell lines with artificial let-7b resulted in a reduced number of proliferating cells (less cells were detected to be in S-phase, accompanied with an increased number of cells in G₁) as well as a strong reduction of the cells' potential to form colonies in soft agar (Schultz et al. 2008).

Almost synchronously, Mueller and Bosserhoff (2008) reported loss of expression of a second member of the let-7 miRNA family – let-7a – to have a potent impact on the invasive potential of melanoma cells. Analyzing regulation of integrin beta3 expression in detail, they found that (in contrast to other cell types) promoter-dependent mechanisms are only subsidiary in melanoma cells whereas the cellular let-7a level is the main determinant of integrin beta3 protein production (Mueller and Bosserhoff 2008). Strikingly, introduction of artificial let-7a molecules into a highly aggressive melanoma cell line lacking endogenous let-7a expression resulted in an about 75% reduced invasive potential *in vitro*. Vice versa, blocking endogenous let-7a in otherwise normal melanocytes using anti-miRs was sufficient to induce migratory behavior in this cell-type (Mueller and Bosserhoff 2008). The authors attributed these effects mainly to let-7a's impact on integrin beta3, as up-regulation of the classical vitronectin receptor alphaVbeta3 has repeatedly been shown to be correlated with malignant potential and aggressive tumor growth in melanoma (Albelda et al. 1990; Danen et al. 1994, 1995; Van Belle et al. 1999). However, like presumably every miRNA known, let-7a targets a cohort of additional transcripts and this fact might strengthen or even potentiate its role in melanoma progression. Mueller and Bosserhoff (2008) themselves highlight the suppression of N-Ras by let-7a, which they used to monitor efficiency of let-7a transfection in their experiments. Although the significance of additional Ras knockdown is uncertain against the background of activating BRAF mutations, it might prove particularly effective in a subset of melanomas harboring activating mutations in N-Ras (see Chaps. 7 and 12).

Corroborating findings in other tumor types, members of the let-7 family of miRNAs hence take on a role as potent tumor suppressors in malignant melanoma as well. The complete set of let-7 target genes still remains to be established to identify the most important mediators of let-7's effects. Nevertheless, already at this point the most intriguing fact about let-7 is its ability to interfere with constituents of several distinct pathways involved in malignant transformation of cells in general and in melanomagenesis in special.

6.2.4

Tumor-Suppressive Role of miR-196a in Melanoma

Another miRNA confirmed to act as a tumor-suppressor in malignant melanoma cells is miR-196a. Thereby, it currently seems that this miRNA exerts much of its function by

regulating the expression of at least two class I homeodomain containing transcription factors, HOX-B7 and HOX-C8 (Braig et al. 2010; Mueller and Bosserhoff 2010a).

The expression of miR-196a has been shown to be strongly reduced in melanoma cell lines as well as in a small set of tissue samples derived from primary cutaneous melanomas and melanoma metastases, respectively (Braig et al. 2010; Mueller and Bosserhoff 2010a). In line with general reports characterizing miR-196a as a potent regulator of HOX gene expression [e.g., (Yekta et al. 2004)], reduced miR-196a levels inversely correlate with strongly enhanced expression of the HOX transcription factors HOX-B7 and HOX-C8 in melanoma cells (Braig et al. 2010; Mueller and Bosserhoff 2010a). 3'UTR reporter assays confirmed that miR-196a is actually capable to directly interact with the HOX-B7 (Braig et al. 2010) and HOX-C8 (Mueller and Bosserhoff 2010a) transcripts thereby preventing their translation into protein.

Due to these efforts, Braig et al. (2010) were able to link loss of miR-196a to over-expression of BMP4 (bone morphogenetic protein 4), an event strongly enhancing the migratory potential of melanoma cells (Hsu et al. 2005; Rothhammer et al. 2005, 2008). In their model proposed, diminished levels of miR-196a cause induction of the transcription factor HOX-B7, which in turn activates bFGF (basic fibroblast growth factor) production. In the end, this results in an enforced expression of BMP4 most likely mediated by transcription factors of the Ets family (Braig et al. 2010). Additionally, Mueller and Bosserhoff (2010a) demonstrated that miR-196a indirectly modulates the expression of several genes potentially involved in melanoma progression by regulating HOX-C8 levels. Many of the HOX-C8 target genes described in literature are related to oncogenic processes like cell adhesion, cytoskeleton remodeling, tumor formation, and invasive behavior of tumor cells (Lei et al. 2005, 2006). The authors demonstrated that at least three of them – *cadherin-11*, *calponin-1* and *osteopontin* – appear to be deregulated in melanoma cells partially due to over-expression of HOX-C8 (Mueller and Bosserhoff 2010a).

In the course of the studies cited, melanoma cell clones were generated which stably re-express miR-196a at a level almost equal to that detected in normal melanocytes (Braig et al. 2010; Mueller and Bosserhoff 2010a). Remarkably, those cells were characterized by an about 50% and 40% reduction in their migratory (Braig et al. 2010) and invasive potential, respectively, as well as by diminished tumorigenicity after subcutaneous injection into nude mice as shown in a preliminary experimental setup (Mueller and Bosserhoff 2010a). Vice versa, treatment of otherwise normal melanocytes with anti-miRs against miR-196a was sufficient to induce migratory behavior in this cell type (Braig et al. 2010).

As true for miR-221/222 and let-7 there is a need to extend the set of known, melanoma-relevant miR-196a target genes to eventually draw a complete picture of the molecules mediating this miRNAs' effects on melanomagenesis. Nevertheless, it currently seems like deregulation of HOX protein levels might well be worth a detailed examination as several studies clearly defined a link between aberrant *HOX* gene expression and cancer development [reviewed in (Abate-Shen 2002; Shah and Sukumar 2010)]. Interestingly, a report highlighting miR-196 family members as metastasis-suppressors in breast cancer (Li et al. 2010) additionally strengthens the tumor-suppressive role of miR-196a in malignant melanoma. In contrast, detection of increased miR-196a levels in other types of tumors when compared to their healthy biological correlates [e.g., (Bloomston et al. 2007; Luthra et al. 2008; Maru et al. 2009; Schimanski et al. 2009)] clearly point toward a tissue

specific role of this miRNA. Considering its targets, the *HOX* genes, to be “tumor modulators” harboring the potential to act as both oncogenes and tumor-suppressor genes depending on the respective tissue context (Abate-Shen 2002) this seems to be rather plausible but adds a further level of complexity to this area of research.

6.2.5

Tumor-Suppressive Role of the miR-34 Family in Melanoma

Recently, several reports conclusively demonstrated that the p53 tumor-suppressor protein directly induces expression of miR-34 miRNA family members and probably exerts a significant portion of its expansive impact on cell-cycle control and regulation of apoptosis through these mediators [reviewed in (Hermeking 2010)]. Accordingly, ectopic expression of miR-34 genes has profound effects on cell proliferation and survival causing cell-cycle arrest in G1 phase, inhibiting colony formation in soft agar, as well as inducing cellular senescence and apoptosis, respectively (Bommer et al. 2007; Chang et al. 2007; Corney et al. 2007; He et al. 2007; Raver-Shapira et al. 2007; Tarasov et al. 2007; Welch et al. 2007). Therefore, genes involved in cell-cycle regulation and apoptosis control are significantly enriched in the large set of confirmed miR-34 targets comprising *Bcl-2*, *CCND1*, *CCNE2*, *CDK2*, *CDK4*, *CREB*, *MET*, *MYC*, *SIRT1*, and many more [for a comprehensive list see (Hermeking 2010)]. Some of the studies defining this potent onco-suppressive role of miR-34 members in various tumor entities also include data regarding melanoma (Lodygin et al. 2008; Lujambio et al. 2008; Migliore et al. 2008). The following paragraph aims to summarize these findings and recombine them with results generated in other cancer types to present a perspective on the possibly far-reaching but yet underestimated influence of miR-34 on melanomagenesis.

In humans, the miR-34 miRNAs are encoded by two different loci with one locus encoding miR-34a and the other locus encoding a common precursor for miR-34b/c. The high similarity shared by all three mature miR-34 forms generated points to an overlapping although not completely identical repertoire of target transcripts. Lodygin et al. (2008) demonstrated that expression of miR-34a is commonly silenced in a variety of tumors as a consequence of promoter hypermethylation. This is also true for melanoma cells as 19 of 44 (43.2%) melanoma cell lines as well as 20 of 32 (62.5%) primary melanomas investigated, displayed hypermethylation of the miR-34a promoter resulting in loss of miR-34a pri-miRNA expression (Lodygin et al. 2008). CpG methylation presumably occurs at a region 100–500bp upstream of the miR-34a transcription start site which also includes the p53 binding site. In contrast, normal melanocytes derived from two different donors did not display *miR-34a* promoter methylation implicating that this mechanism represents a tumor-specific event. Interestingly, miR-34a is encoded at chromosome 1p36 – a chromosomal locus repeatedly described to harbor a gene for melanoma susceptibility which surprisingly has not been identified to date [e.g., (Ng et al. 2008; Poetsch et al. 2003)]. LOH for 1p36 had been detected in 77% of nodular melanomas, 86% of metastatic melanomas, but only 20% of superficially spreading melanomas (Poetsch et al. 2003). Additionally, in neuroblastoma a correlation between 1p36 loss and miR-34a down-regulation had been confirmed (Wei et al. 2008). It is therefore tempting to speculate, that in melanoma cells

either loss of 1p36, hypermethylation of the miR-34a promoter or a combination of both mechanisms may result in loss of miR-34a and its tumor-suppressive function.

CpG island methylation had also been found to be the cause for silencing of miR-34b/c in melanoma and several other types of cancer (Kozaki et al. 2008; Lujambio et al. 2008; Toyota et al. 2008). Thereby, the study of Lujambio et al. (2008) strikingly unraveled a highly significant correlation between *miR-34b/c* promoter hypermethylation and the presence of lymph node metastases in a panel of melanoma tissue samples. A report published by Migliore et al. (2008) implicates that this might be due to the release of c-MET expression from miR-34b/c mediated repression. Performing a bioinformatical search for miRNAs targeting c-MET they found miR-34b/c as well as miR-199* to be able to suppress c-MET production and therefore the ability of cancer cells to respond to HGF (hepatocyte growth factor) stimulation – even if they harbored c-MET gene amplification or over-expression resulting in high levels of constitutively active receptor (Migliore et al. 2008). In their study, melanoma-derived primary cells expressed only limited amounts of miR-34c (and therefore possibly also of miR-34b). Transfection of synthetic miR-34b/c molecules into these cells led to down-regulation of c-MET expression as well as to impairment of c-MET-mediated motility *in vitro* (Migliore et al. 2008). The *MET* oncogene encodes a tyrosine kinase receptor which upon activation by binding its ligand HGF (also called scatter factor) activates a complex biological program which in the end results in invasive growth and metastatic dissemination of tumor cells (Birchmeier et al. 2003). In melanoma, over-expression of c-MET has repeatedly been reported and its transcriptional activation had been linked to increased levels of SOX10, PAX3 and MITF whereas loss of Plexin B1 prevents suppression of c-MET activation by absent receptor-receptor interactions [e.g., (Cruz et al. 2003; Kiriakidou et al. 2004; Mascarenhas et al. 2010; Puri et al. 2007)]. It is known from other tumor types that c-MET expression can also be induced during hypoxia [through binding of HIF-1 α to the *MET* promoter; (Pennacchietti et al. 2003)] or activation of several oncogenes including *Ets* family members (Gambarotta et al. 1996; Ivan et al. 1997; Webb et al. 1998). While HGF is a paracrine mitogen for normal melanocytes, most melanoma cells gain the potential to produce HGF themselves thereby establishing sustained autocrine stimulation of the receptor (Li et al. 2001). Notably, correlations between c-MET expression and the metastatic potential of melanoma cells as well as patient survival have been described (Barnhill and Mihm 1993; Natali et al. 1993; Slominski et al. 2001).

These findings implicate that loss of expression of miR-34 family members – potentially caused by multiple mechanisms (including aberrant methylation and chromosomal aberrations) – might be a central event in the progression of primary melanomas on to invasive and metastatic disease. They may additionally shed new light on the controversially disputed role of p53 in melanomagenesis. Whereas silencing of p53 is acknowledged to be one of the most influential events in the formation of virtually all tumor types, its role in melanoma has been neglected due to a low frequency of missense mutations detected [0–10%; reviewed in (Box and Terzian 2008)]. However, as indicated by several studies performed in animal models, the impact of p53 on melanomagenesis should be revisited; e.g., Terzian et al. (2010) recently demonstrated that high levels of p53 prevent the conversion of nevi into malignant and metastatic melanomas. Some of the conflicting effects observed might be explained by alterations in the expression of miR-34 family members impairing p53's ability to maintain its tumor suppressive function in advanced melanomas.

That is, their rapidly growing *TP-ras*^{0/+} tumors (Terzian et al. 2010) might overcome the tumor-suppressive effect of high-level stabilized wild-type p53 by losing miR-34 expression. In summary, these observations might also harbor some exciting insights into a key aspect contributing to the pronounced chemotherapy resistance of melanoma (Soengas and Lowe 2003) as loss of miR-34 expression had been linked to resistance against apoptosis induced by p53 activating agents (Zenz et al. 2009).

6.2.6

Other miRNAs with a Functional Impact on Melanomagenesis

6.2.6.1

miR-193b

Profiling the expression of 470 miRNAs in a set of formalin-fixed paraffin-embedded (FFPE) specimens derived from eight benign nevi and eight metastatic melanomas Chen et al. (2010) detected 31 miRNAs to be differentially expressed in the melanoma samples (13 up-regulated, 18 down-regulated miRNAs). The authors selected the miRNA most strongly down-regulated, miR-193b, for a more detailed functional analysis. Introduction of artificial miR-193b molecules into three different melanoma cell lines resulted in a reduction of their proliferative capacity of between 30% and 60% marked by an increased number of cells in G1 phase whereas the fractions of cells detected to be in S phase or G2 phase decreased (Chen et al. 2010). Combining whole genome cDNA microarray profiling and bioinformatic miRNA target prediction the authors identified cyclin D1 (CCND1) as a potential target for miR-193b. Subsequent 3'UTR reporter assays confirmed miR-193b to be a further miRNA targeting CCND1 in melanoma – in addition to let-7b (see above).

6.2.6.2

miR-532-5p

Members of the Runt-related (RUNX) family of transcription factors function as scaffolds interacting with co-regulatory factors often involved in tissue differentiation (Javed et al. 2005). Driven by findings on a tumor-suppressive role of RUNX3 in several types of tumors, Kitago et al. (2009) detected a significant down-regulation of this transcription factor on mRNA level in their set of 82 primary melanomas and 41 melanoma metastases as compared to 12 normal skin samples. Thereby, RUNX3 down-regulation displayed a nonlinear association with AJCC stage of the tumors with AJCC stage IV classified metastatic melanomas showing an even stronger reduction of RUNX3 expression compared to primary tumors. Interestingly, multivariate analysis revealed that down-regulation of RUNX3 mRNA was related to disease outcome in those patients (Kitago et al. 2009). In contrast to tumors derived from other tissues (Ito 2004), loss of RUNX3 expression was not due to promoter hypermethylation in the melanoma samples but seems to be associated with increased expression of miR-532-5p. Kitago et al. (2009) detected this miRNA to be significantly over-expressed in their metastatic cell lines compared to melanocytes as well

as in the metastatic melanoma samples compared to primary tumors pointing toward an enforced expression of miR-532-5p as progression to metastatic disease occurs. While transfection of anti-miRs against miR-532-5p into a metastatic melanoma cell line resulted in increased expression of RUNX3 on mRNA and protein level, a direct interaction of the miRNA with a target site in the RUNX3 3'UTR has not yet been confirmed. Another interesting fact is that miR-532-5p is actually encoded at Xp11.23 which is located next to Xp11.3 – the genomic locus encoding miR-221/222 (see above).

6.2.6.3 miR-155

Levati et al. (2009) analyzed several miRNAs known to be involved in oncogenic processes for their expression in a panel of melanoma cell lines. In the majority of cell lines used they detected an enhanced expression of miRNAs belonging to the miR-17-92 cluster – miR-17-5p, miR-18a, miR-20a, and miR-92a – as well as a diminished expression of miR-146a, miR-146b, and miR-155 compared to normal melanocytes (Levati et al. 2009). As miR-155 down-regulation was most prominent, the authors decided to examine this miRNA's impact on melanoma cell function. Transfection of artificial miR-155 molecules into melanoma cells inhibited proliferation by between 30% and 98% in 12 out of 13 cell lines tested. Subsequent experiments performed in four of the cell lines implicated that this impairment of melanoma cell proliferation was at least partly based on induction of apoptosis in miR-155 transfected cells (Levati et al. 2009). Reports on other types of tumors point toward a tissue specific function of miR-155 ascribing the latter oncogenic as well as a tumor-suppressive potential depending on cellular context [e.g., (Gartel and Kandel 2008; Volinia et al. 2006)]. The functional impact of miR-155 down-regulation on melanomagenesis remains to be defined as it appears to be only weakly expressed already in normal melanocytes. Additionally, it seems like loss of miR-155 expression is a cell culture-related phenomenon so that this miRNA might rather be up-regulated during melanoma progression *in vivo* [(Philippidou et al. 2010; Segura et al. 2010); also see Sect. 6.3].

6.2.6.4 miR-210

Zhang and co-workers reported a correlation between elevated pri-miR-210 levels in melanoma tissues and shorter metastasis-free survival of the patients in Kaplan–Meier analysis (Zhang et al. 2009). MiR-210 is the miRNA species most prominently induced during hypoxia [reviewed in (Huang et al. 2010)]. In their study, Zhang et al. (2009) demonstrated that over-expression of this miRNA overrides hypoxia-induced cell-cycle arrest by indirectly activating the cell cycle and metabolic regulator MYC via repression of its antagonist MNT in several tumor cell lines. It was therefore hypothesized that miR-210 is involved in regulation of mitochondrial biogenesis and central carbon metabolism to adopt tumor cells to hypoxic episodes and to further provide them a competitive advantage in

developing tumors once normal oxygen levels are restored (Morrish 2009). Hypoxia and HIF signaling have also been shown to be implicated in melanomagenesis and melanoma metastasis [reviewed in (Bedogni and Powell 2009)], especially as a potential constitutive HIF-1 activity has been described for melanoma (Kuphal et al. 2010). Unfortunately, besides the Kaplan–Meier analysis cited above no data regarding melanoma cells is provided by Zhang and colleagues which performed all their experiments in cell lines derived from other tumor types. Though, Satzger et al. (2010) detected significantly enhanced expression of miR-210 in 16 primary melanoma samples as compared to 11 melanocytic nevi investigated. However, miR-210 expression did not correlate with recurrence-free survival or overall survival in an extended set of 112 primary tumors (Satzger et al. 2010). Additionally considering a currently disputed bipolar effect of hypoxia and miR-210 on tumorigenesis (Huang et al. 2009) a lot more research will be necessary to identify the molecular interactions of the HIF/miR-210 network with relevance to melanoma as well as to define the specific impact of miR-210 on the progression of this disease.

6.3

Lessons to Learn from miRNA Expression Profiling in Malignant Melanoma

In addition to analyses focusing upon the functional impact of single miRNA species on melanoma progression, several studies performing global miRNA expression profiling or determining expression patterns of miRNA subsets in melanoma cell lines and tissue samples have been conducted. In the beginning, this topic was only addressed as a part of large scale profiling studies in which few melanoma samples had been included [(Blower et al. 2007; Gaur et al. 2007; Lu et al. 2005; Zhang et al. 2006); reviewed in (Mueller and Bosserhoff 2009)]. Therefore, it had been difficult to deduce information on alterations in the miRNA profile of melanoma cells clearly distinguishing them from normal melanocytes with only these data published.

From 2008 on research specifically aimed to identify differences in the miRNA expression patterns of normal melanocytes, nevi and melanoma cells. Objective was to either identify single deregulated miRNAs as targets for in-depth functional characterization (Chen et al. 2010; Molnar et al. 2008; Mueller et al. 2009; Philippidou et al. 2010; Schultz et al. 2008) or to identify potential melanoma biomarkers (Caramuta et al. 2010; Glud et al. 2009; Jukic et al. 2010; Leidinger et al. 2010; Liu et al. 2009; Ma et al. 2009; Segura et al. 2010). Further, some groups analyzed limited sets of miRNAs already known to be involved in tumorigenic mechanisms to confirm their deregulated expression in melanoma as well (Levati et al. 2009; Satzger et al. 2010). Another experimental approach utilized next generation sequencing to potentially determine the complete miRNAome of the melanocytic lineage [(Stark et al. 2010); discussed in detail in (Mueller and Bosserhoff 2010b)].

The data obtained during these studies can be exploited in different ways. On the one hand, deregulated expression of miRNAs which had already been functionally characterized in melanoma before can be confirmed in larger sets of cell lines or tissue samples as well as new candidates for functional analysis can be identified depending on the degree of

deregulation detected. On the other hand, miRNA classifiers suited for diagnostic and/or prognostic purposes could be created – based less presumably on a single miRNA but rather on a larger set of miRNAs.

6.3.1

Confirmation of Deregulated Expression of Single miRNAs and Identification of New miRNA Candidates for Functional Characterization

Misexpression of many of the miRNAs discussed in Sect. 6.2 was confirmed in independent sample sets during profiling studies highlighting their importance in melanoma progression (data included in Table 6.1). For example, down-modulation of let-7a in melanoma tissues has repeatedly been detected. In summary, those findings point to a model in which normal skin melanocytes as well as benign nevi show high expression of let-7a which then successively gets diminished during melanoma progression with melanoma metastases displaying the lowest let-7a levels (Chen et al. 2010; Glud et al. 2009; Ma et al. 2009; Philippidou et al. 2010). Further, a reduced expression of let-7b (Chen et al. 2010) and miR-196a (Caramuta et al. 2010; Philippidou et al. 2010) as well as an enhanced expression of miR-221/222 (Mueller et al. 2009; Philippidou et al. 2010) has concurrently been observed in different sets of melanoma cell lines and tissue samples. These are examples of positive correlations indicating that miRNA profiling studies can clearly be utilized to support and strengthen findings obtained during functional studies which are usually performed *in vitro* in rather small sets of cell lines. However, microarray data should be taken into account with care unless validated by qRT-PCR or northern blotting.

Despite consistent results have been obtained in regard to specific and prominent aberrations in miRNA expression, there are also some discrepancies and the overall correlation between independent miRNA profiling studies in melanoma is rather poor. For instance, Caramuta et al. (2010) reported let-7b to be up-regulated in their melanoma specimens and Satzger et al. (2010) did not find miR-222 significantly up-regulated in their set of tissue samples. Further, Caramuta et al. (2010) detected reduced levels of miR-15b in melanoma cells – in sharp contrast to Satzger and co-workers (2010) who detected miR-15b to be strongly up-regulated (see below). Adding to the confusion, a highly pronounced variation is observed in the expression levels of single miRNAs between melanoma cell lines and tissue samples, respectively (Caramuta et al. 2010; Philippidou et al. 2010; Satzger et al. 2010). As an example, Segura et al. (2010) found expression of miR-155 rather up-regulated in melanoma lesions compared to benign nevi which is in stark contrast to the functional report published by Levati et al. [(2009), see above]. This caveat might be resolved by the findings from Philippidou et al. (2010) who detected miR-155 to be down-regulated in melanoma cell lines compared to normal melanocytes, but up-regulated when comparing melanoma tissue samples to benign nevi.

This amongst other things indicates that interpretation of data obtained during miRNA profiling studies is heavily depending on the type of sample used as baseline control. As isolated melanocytes show a high degree of variation in their miRNA expression profiles not only dependent on donor source but also on passage number (as already known from their cDNA and protein profiles), each study should include an extended set of

melanocytes derived from different donors and intervals of propagation in cell culture should be adequately limited. Interestingly, the miRNA profiles generated from tissue samples seem to overlap stronger between independent studies than do expression profiles generated from cell lines [e.g., (Philippidou et al. 2010) compared to (Glud et al. 2009)]. However, when analyzing tissue specimens most commonly benign nevi are used as baseline control and therefore serve as biological correlate for melanoma cells. Undoubtedly, nevi cells cannot be considered equivalent to normal skin melanocytes as they already harbor potentially malignant genetic alterations rendering them abnormal by definition [for a detailed discussion see (Mueller and Bosserhoff 2010b)]. Additionally, tissue samples are prone to contamination by non-melanocytic cells derived from the surrounding tumor stroma. Therefore, microdissection should be established a standard in sample preparation in order to obtain reliable results.

Of note, not only the baseline control will influence interpretation of miRNA profiling data but so will data processing. Although challenging, a consensus must be aspired on normalization of miRNA microarray data as well as on a common stable reference gene for qRT-PCR based miRNA profiling methods [reviewed in (Meyer et al. 2010)]. Moreover, as next generation sequencing setups find their way into everyday laboratory use [see also (Stark et al. 2010) and related discussion in (Mueller and Bosserhoff 2010b)] the general debate on which is the best method for miRNA expression profiling is fueled further [reviewed in (Git et al. 2010)].

The high variability observed in the profiling studies cited above indicates that miRNAs consistently detected to be deregulated in independent sample sets might be best candidates to actually playing a central role in melanomagenesis. And indeed, some miRNAs meet this claim. The most prominent example is miR-17-5p which together with other members of the well-characterized oncogenic miR-17-92 cluster [reviewed in (Olive et al. 2010)] has been reported to be up-regulated in melanoma cells (Chen et al. 2010; Levati et al. 2009; Mueller et al. 2009; Philippidou et al. 2010). Interestingly, Levy et al. (2010) recently hypothesized that miR-17 mediated suppression of the proapoptotic BIM protein may play a significant role in survival of melanocytic cells in the absence of Bcl-2. Further, reduced expression of miR-200 family members which play a major role in epithelial-to-mesenchymal transition [reviewed in (Bracken et al. 2009)] had concordantly been demonstrated by several groups indicating a role of these miRNAs in the progression to advanced melanoma as well (Chen et al. 2010; Philippidou et al. 2010; Schultz et al. 2008). Down-regulation of miR-194 (Caramuta et al. 2010; Mueller et al. 2009) and miR-211 (Caramuta et al. 2010; Chen et al. 2010; Jukic et al. 2010) as well as up-regulation of miR-210 (Caramuta et al. 2010; Philippidou et al. 2010) and miR-373 (Mueller et al. 2009; Philippidou et al. 2010) are further examples for deregulated expression of miRNAs not yet analyzed for their specific functions in malignant melanoma. Thereby, the role of miR-373 in melanoma might be more sophisticated than in other types of tumors (Mueller et al. 2009; Satzger et al. 2010).

This implicates that a lot more miRNAs might be functionally involved in the formation and progression of malignant melanoma than the few we started to analyze in detail so far. Fortunately, a reasonable amount of miRNA profiling data is now available to support the identification of candidate miRNAs for future functional validation. Nevertheless, for every single miRNA it remains to be elucidated if its deregulated expression really is a

defect driving melanoma progression or if it just has to be considered a passenger defect not reflective of the fundamental molecular mechanisms underlying tumorigenesis.

6.3.2

Identification of miRNA Biomarkers Suitable for Diagnostics and Prognostics in Melanoma

In the beginnings of cancer-related miRNA research groundbreaking publications demonstrated that (1) many miRNAs are encoded at genomic loci matching to fragile sites or regions associated with cancers (Calin et al. 2004) as well as that (2) miRNA expression profiles reflect the developmental lineage and the differentiation state of solid tumors (Lu et al. 2005; Volinia et al. 2006). Strikingly, poorly differentiated tumors can be successfully classified by their miRNA expression profile, in contrast to their mRNA profile – leading Rosenfeld and colleagues to construct a 48 miRNA classifier capable to determine the origin of metastatic tumors of unknown primary origin with high accuracy (Gaur et al. 2007; Lu et al. 2005; Rosenfeld et al. 2008). With regard to melanoma, these studies unrevealed that 85.9% of genomic loci harboring one or more of 283 examined miRNA genes exhibited DNA copy number alterations and that some of these changes were specific to this tumor type (Zhang et al. 2006). In addition, a set of 15 miRNAs expressed at significantly different levels separated the eight melanoma cell lines included in the NCI-60 panel from the other cancer cell lines investigated (Gaur et al. 2007). Taken together, these findings paved the way to explore the potential of miRNA expression profiling to identify miRNA biomarkers with diagnostic and/or prognostic value in malignant melanoma.

Fortunately, in accordance to other tumor tissues FFPE specimens derived from melanocytic lesions are suitable starting material for miRNA expression profiling (Glud et al. 2009; Liu et al. 2009; Ma et al. 2009). In the light of a very limited availability of fresh-frozen melanoma samples (due to therapeutic guidelines requiring microscopic analysis of the whole primary tumor to determine histopathological prognostic parameters, including Breslow thickness), as well as of the large quantity of archival material stored in pathological institutes worldwide this really opens avenues for miRNA biomarker discovery. First interesting results already point out that miRNA profiles actually do harbor the potential to be utilized in diagnostics and prognostics of melanoma in the future. In this way, Satzger and colleagues (2010) found significant association of high miR-15b expression with poor recurrence-free survival and overall survival in Kaplan–Meier analysis of a total of 128 melanoma patients (median follow-up 43.1 month). They also showed that up-regulation of miR-15b was a statistically significant independent parameter of disease-free survival and overall survival in multivariate Cox analysis in addition to Breslow thickness and ulceration of the primary tumor (Satzger et al. 2010). In addition, Caramuta et al. (2010) described low levels of miR-191 and high levels of miR-193b in melanoma lymph node metastases to be associated with shorter survival after diagnosis of metastatic dissemination into the regional lymph nodes analyzing 16 melanoma patients.

In contrast to these studies focusing on single miRNA markers associated with melanoma survival, Segura et al. (2010) constructed a miRNA classifier consisting of six miRNAs (miR-150, miR-342-3p, miR-455-3p, miR-145, miR-155 and miR-497) and indicative for melanoma prognosis. Based on this classifier they were able to predict post-recurrence

survival in their set of 59 FFPE melanoma metastases (derived from patients with detailed clinical follow-up) with an estimated accuracy of about 80%. Notably, the miRNA classifier was also able to significantly risk-stratify stage III melanoma patients into “better” and “worse” prognostic categories based on survival probability in contrast to the AJCC standard classification system [stages IIIB and IIIC; (Segura et al. 2010), detailed discussion in (Mueller and Bosserhoff 2010b)]. While the reports cited above rely on miRNA expression profiling in tissue samples Leidinger et al. (2010) analyzed blood cell miRNA profiles in 35 melanoma patients and 20 healthy control individuals. Utilizing a subset of 16 significantly regulated miRNAs they were able to separate melanoma patients from healthy individuals with an accuracy of 97.4%, a specificity of 95% and a sensitivity of 98.9% (Leidinger et al. 2010). This study provides first evidence that miRNA expression profiles generated from peripheral blood cells could be engaged to distinguish patients suffering melanoma from healthy individuals in a noninvasive routine diagnostic approach – although it is not yet clear which cells in the blood are actually responsible for differences in the miRNA signatures obtained.

As true as for other cancers where tumor markers for early neoplastic transformation remain to be discovered, melanoma biomarkers distinguishing between nevi and early melanoma are urgently needed – especially considering the ability to metastasize a very early event in the progression of malignant melanoma. MicroRNA biomarkers hopefully not only suite this purpose but may also be utilized for separating primary melanomas which will lead (or have already led) to metastasis from non-metastasizing tumors. Regarding prognostic applications of miRNAs, a central goal has to be the identification of miRNA classifiers (which are representative of molecular biomarkers in general) that can subsequently be incorporated into established staging systems relying solely on morphological criteria. In the end, this will hopefully allow better separation of patients into treatment groups based on their risk and prognosis. Thereby, patients responsive to one kind of adjuvant treatment could be identified while nonresponders could be spared negative effects of treatment. However, for prognostic as well as diagnostic applications, large-scale studies in comprehensive sets of tissue or blood samples (for which detailed clinical data have to be available) are needed ultimately to prove the usefulness of single miRNAs or miRNA classifiers which already have been proposed as melanoma biomarkers to date. Taken together, although miRNA analysis is unlikely to replace the existing tools for tumor diagnosis and management (like immunohistological staining or established serum marker-proteins), miRNA biomarkers promise huge benefits if used to complement established methodologies.

6.4 Conclusion

Undoubtedly, a sense of euphoria encases the scientific community when considering future therapeutic and biomarker applications based on miRNAs. This attitude is strengthened as first diagnostic kits (Asuragen Inc.; Rosetta Genomics) made their way into commercial application and a first miRNA-based drug entered phase IIa clinical trials in the US

and Europe (Santaris Pharma A/S) – with some equally promising miRNA therapeutics subjected to tests in preclinical studies (Regulus Therapeutics Inc.; Mirna Therapeutics Inc.). While we by now successfully demonstrated that miRNAs are new players in melanomagenesis we are yet far away from a complete understanding of the impact those tiny molecules exert on formation and progression of this malignancy. Undoubtedly, expanding our knowledge on melanoma-relevant miRNAs will be an indispensable prerequisite to develop therapeutic approaches based on miRNA to eventually allow for the effective treatment of melanoma patients in the future. Nevertheless, already at this point it seems like miRNAs could at least fill some of the gaps in our yet limited knowledge of the molecular mechanisms underlying this deadly disease. A beginning has been made – a long but promising way is still ahead.

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Abstract Signal transduction pathways are central in any cellular biological process, as they provide the actual signals between extra or intracellular stimuli and protein kinases, transcription factors, and other regulatory proteins. Naturally, this is the most important component in control of any cellular process, and therefore it is not surprising that it is often deregulated in cancer. Melanoma is in fact a paradigm for rewired signaling, since most critical mutations discovered in this tumor type are centered around a few major signaling cues, the MAPK and PI3K pathways. By their nature, these regulatory components harbor catalytic activities, and therefore are preferred targets for therapy. Here we summarize the current knowledge of major signaling pathways that are deregulated in melanoma and the implication of such deregulation for the biology of this tumor.

7.1

Extracellular Receptors

Among the receptors that were reported to be deregulated in melanoma are numerous membrane-bound G protein-coupled receptors or receptor tyrosine kinases, including MC1R, c-Kit, c-Met, IGFR, and Frizzled (Fargnoli et al. 2010; Landi et al. 2006; Mattei et al. 1994; Topcu-Yilmaz et al. 2010).

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proliferation (Hunt et al. 1995; Mountjoy et al. 1992; Robinson and Healy 2002; Suzuki et al. 1996).

MC1R exhibits genetic variance, with hot spot mutations at residues R151C, R160W, and D294H – that reduce receptor function and result in fair skin, freckled, and red hair phenotype (Kadekaro et al. 2003). These specific mutations are associated with reduced UV-induced DNA damage repair efficiency and increased melanoma risk (Scott et al. 2002; Song et al. 2009). Specifically, stimulation of MC1R by α -MSH potentiates p16INK4A after UV exposure (Pavey et al. 2002). In contrast, overexpression of MC1R variants has been shown to render cells insensitive to α -MSH-mediated suppression of cell proliferation (Robinson and Healy 2002), suggesting that polymorphic variants of MC1R may enhance melanoma susceptibility or progression at least in part by attenuating p16INK4A function.

Although MC1R is neither genetically nor epigenetically silenced (Kim et al. 2008a), expression of its mutant, functionally impaired variants compromises adequate MC1R function, and furthermore correlates with increased melanoma risk (Landi et al. 2006). MC1R variant carriers exhibit higher melanoma risk in the presence of CDK2NA mutations (Fargnoli et al. 2010). Notably, germline mutations of MC1R in melanoma were associated with increased risk incidence of B-Raf mutations (Landi et al. 2006).

Recent studies demonstrate that mutation of G proteins themselves, in particular α -subunit Q (GNAQ), also may exhibit alterations in early melanoma lesions (Kusters-Vandeveldel et al. 2009; Lamba et al. 2009; Van Raamsdonk et al. 2009). GNAQ is mutated within a Ras-like domain at position Q209L. This mutation renders the protein constitutively active, resulting in enhanced PKC and MAPK signaling. Overexpression of GNAQ Q209L mutant is sufficient to confer anchorage independence and increased tumorigenicity of immortalized melanocytes.

MC1R, c-Kit, c-Met, and IGFR are amongst growth factor receptors that are deregulated in melanoma, and contribute to the upregulation of Ras-MAPK and PI3K-Akt signaling axes despite the presence of downstream Ras mutations. Stem cell factor (SCF), hepatocyte growth factor (HGF), and Insulin-like growth factor (IGF) are the ligands for c-Kit, c-Met, and IGFR, respectively, and binding to their cognate receptors results in parallel activation of downstream Ras-Raf-Mek-MAPK and PI3K-Akt pathways, which promote cell survival and proliferation. Albeit not necessarily found in a uniform fashion across melanomas, altered expression of these receptors in melanoma has been implicated in tumor phase- or tissue-type specificity (receptor-specific details below).

7.1.2

c-Kit

c-Kit expression is apparent in early phase or radial growth melanomas. Although the penetrance appears to be low, c-Kit activating mutation, L576P, has been reported in melanoma (Antonescu et al. 2007; Rivera et al. 2008; Willmore-Payne et al. 2006). Interestingly, however, downregulation of its expression is associated with melanoma progression (Giehl et al. 2007; Janku et al. 2005; Montone et al. 1997; Natali et al. 1992). These observations suggest that initial upregulation of c-Kit, as well as its ligand, SCF, may be required for

establishment of early primary lesions, but that its continued expression is not needed during invasion and metastasis. The disparity of mutations in or altered expression of c-Kit amongst different melanoma was initially overlooked in clinical trials employing c-Kit-specific inhibitors. More recent trials observed clinical efficacy when patient cohorts harboring c-Kit mutation were treated with the highly selective pharmaceutical inhibitor, Gleevec (Terheyden et al. 2010). How the inhibition of c-Kit contributes to the melanoma progression remains an important topic for investigation. It is proposed that as SCF is a keratinocyte-secreted factor, and downregulation of its cognate receptor by melanoma cells may allow them to escape SCF-mediated cell death. Indeed, *in vitro* studies demonstrate that reexpression of the c-Kit receptor in metastatic melanoma and subsequent exposure to SCF sensitizes these cells to SCF-mediated apoptosis and reduction of their tumorigenic and metastatic potential *in vivo* (Bar-Eli 1997; Huang et al. 1996; Willmore-Payne et al. 2005).

Although how c-Kit is downregulated during melanoma progression remains rather unclear, a recent study demonstrates that epigenetic downregulation of c-Kit may be linked with expression of microRNAs (see also Chap. 6), specifically miR221 and miR222, which were shown to suppress expression of both c-Kit and p27Kip (Felicetti et al. 2008).

7.1.3

c-Met

c-Met-dependent signaling is also amplified in melanoma, although genetic mutations or modifications have not appeared to be a prevalent phenomenon that result in aberrant activation of c-Met. A recent study has identified two c-Met mutations in melanoma cell lines and tumor tissues: N948S and R988C, which confer greater activity for c-Met signaling, as measured by its downstream effectors, including MITF, tyrosinase, as well as AKT and its effectors (Chin et al. 2006; Puri et al. 2007a). Nonetheless, c-Met exhibits upregulation, particularly in later-stage melanoma (Natali et al. 1993) which has been implicated in metastasis, especially to the liver (Rusciano et al. 1995).

Upregulated c-Met can stem from a number of perturbations. c-Met is regulated by Microphthalmia-Associated Transcription Factor (MITF), which is activated by α -MSH (Rusciano et al. 1999). As mentioned above, α -MSH binds and activates its receptor, MC1R, which drives the signaling cascade to upregulate MITF (Rouzaud et al. 2006). Impaired MC1R function, which is common in many melanomas, is indicative of deregulated c-Met at the genetic and protein levels.

Another component implicated in the regulation of cMet is skeletotrophin, a ubiquitin ligase that is lost in melanoma due to increased SNAIL-mediated transcriptional repression. Reexpression of Skeletotrophin in melanoma cells impairs the cells' invasive capacity *in vitro*, and correlates with a reduction in c-Met mRNA transcripts (Takeuchi et al. 2006). In addition, abrogated microRNA has also recently been demonstrated to contribute to increased c-Met levels in melanoma. Mir34a is a microRNA that is expressed in melanocytes, but that is lost in melanoma, and its reexpression *in vitro* can reduce c-Met expression, suppress melanoma cell growth, as well as its migration and invasion capacities (Yan et al. 2009).

7.1.4

IGFR1

Insulin-like growth factor receptor (IGF1R) has been identified as another growth factor receptor that is upregulated in progressively malignant melanoma (Mallikarjuna et al. 2006). In early melanoma lesions, IGF1R appears to enhance cellular growth and survival by promoting MAPK- and β -catenin-dependent signaling pathways, although IGF1R-dependent stimulation of these two pathways may be dispensable in later stage melanomas, when other oncogenes are constitutively active (Satyamoorthy et al. 2001).

Upregulation of IGF1R is associated with both malignant progression and resistance to apoptotic stimuli. Antisense-mediated inhibition of IGF1R is sufficient to inhibit the growth of mouse melanoma cells in nude mice (Resnicoff et al. 1994), and monoclonal antibody-targeted inhibition of IGF1R in human melanoma cells in xenograft mouse models shows similar inhibition of growth and invasion (Maloney et al. 2003). Furthermore, disruption of IGF1R can sensitize melanoma cells to TRAIL induced-apoptosis (Karasic et al. 2010), and can also increase radiosensitivity of melanoma by impairing ATM-mediated DNA damage response (Macaulay et al. 2001). In human melanoma, inhibition of IGF1R is moreover sufficient to suppress melanomas harboring B-Raf^{V600E} mutation (details below), indicating that inhibition of IGF1R can override downstream signaling that circumvents the known IGF1R effector, Ras-MAPK signaling axis (Yeh et al. 2006).

7.1.5

WNT- β -Catenin

The WNT- β -catenin signaling pathway regulates melanocyte development and is deregulated in melanoma (see also Chap. 5 and 7). WNT is a secreted ligand for its membrane receptor, Frizzled (Fig. 7.2). WNT binding to Frizzled results in Frizzled's activation of the cytoplasmic effector, Dishevelled. Consequently, Dishevelled inhibits GSK3 β -Axin-APC-mediated degradation of β -catenin, allowing the stabilization and nuclear import of β -catenin to execute its transcriptional functions. Activation of the WNT- β -catenin pathway facilitates β -catenin-mediated upregulation of MITF that promotes melanocyte differentiation and development (Dorsky et al. 2000; Takeda et al. 2000). MITF can itself bind to β -catenin, tilting transcriptional activity toward MITF targets and generating a positive feedback loop (Schepsky et al. 2006).

Indeed, melanoma tumors harboring activating- β -catenin mutations also exhibit upregulation of MITF (Doglioni et al. 2003). Interestingly, β -catenin also upregulates the Brn-2 transcription factor, which transcriptionally represses MITF while simultaneously enhancing invasive melanoma behavior, and which also characterizes distinct subsets of melanoma cells that are MITF-negative (Goodall et al. 2008). The implication of MITF heterogeneity within the same and different tumors is subject to intense investigation.

WNT- β -catenin signaling is upregulated in melanoma, and although only ~3% of melanoma biopsies are observed to harbor mutations in β -catenin, ~30% of human melanoma nonetheless exhibit increased nuclear β -catenin (Larue and Delmas 2006). Upregulation of WNT signaling directly upregulates MITF and Brn-2 with concomitant suppression of

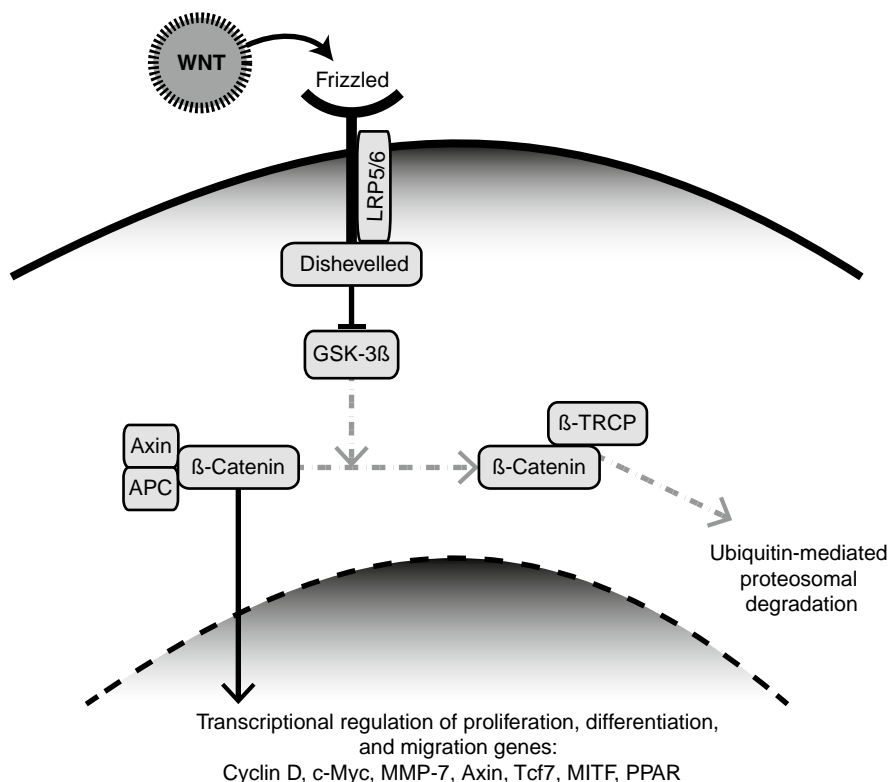


Fig. 7.2 Wnt signaling pathway. Binding of Wnt to its cognate receptor and co-receptor, Frizzled and LRP5/6, results in inactivation of GSK3 β via Dishevelled. Inactivation of Dishevelled stabilizes β -Catenin by releasing it from its degradation complex that includes Axin and APC. During non-stimulated conditions, β -Catenin is bound by β -TRCP, which facilitates its ubiquitination and subsequent proteosomal-degradation. Stabilized β -Catenin can then be imported into the nucleus to facilitate the transcriptional regulation of proliferation, differentiation, and migration genes. In melanoma, upregulated branches of this signaling pathway are indicated in *black*; downregulated branches are indicated by *dashed grey arrows*

p16INK4A transcription – a concerted effort to drive melanoma growth and proliferation (Delmas et al. 2007; Goodall et al. 2004a; Widlund et al. 2002).

Interestingly, the functional role of WNT3, WNT signaling in melanoma development depends on the specific WNT isoform. WNT3, an activator of the canonical Wnt signaling axis, is anti-tumorigenic, correlating with primary/nevi lesions, and decreased proliferation *in vitro* and *in vivo* tumor models. Furthermore, WNT3 correlates with upregulation of melanocyte development and differentiation genes, including Axin, Tcf7, and MITF (Chien et al. 2009). In contrast, WNT5a, an activator of the noncanonical Wnt signaling axis, appears to antagonize the transcriptional effects of WNT3A. Indeed, WNT5a is pro-tumorigenic, cooperating with other signaling pathways (e.g., PKC) to enhance metastatic and invasive behavior of melanoma cells, likely via its known function

in redistribution of adhesion receptors (Weeraratna et al. 2002; Witze et al. 2008). Indeed, melanoma tumors that stain positive for WNT5A appear to exhibit increased invasiveness and decreased proliferation, compared to counterparts that are WNT5A-negative but MITF and Melan-A positive, supporting a model of proliferative vs. invasive isotype switching during tumor progression (Eichhoff et al. 2010).

7.2

MAPK Signaling Axis

Altered expression or activity of the above membrane receptors (MC1R, c-Kit, c-Met and IGFR, and WNT) comprises the most external level of perturbed signaling cues that can promote melanoma formation and progression. Downstream of these receptors are the effector-signaling pathways, several of which are themselves deregulated in melanoma.

Among these, the majority of melanomas constitute deregulated MAPK signaling, due to mutation in upstream N-Ras or B-Raf genes. As a consequence, downstream kinases and transcription factors are rendered constitutively active, regardless of upstream aberrations. In this section we will review our current understanding of pathways along the commonly altered MAPK axis, and the implication toward melanoma development or progression.

The MAPK pathway is coupled to the upstream membrane receptors by the Ras-family of small G-proteins: H-Ras, K-Ras, and N-Ras (Bos 1989; Dhillon et al. 2007). In non-transformed cells, these Ras proteins are responsive to upstream membrane receptor activation (i.e., c-Kit, c-Met and IGFR, and WNT), transducing activating signals further through interplay with the Raf-family of effector serine/threonine kinases: A-Raf, B-Raf, and C-Raf. Signals are then transduced down a cascade of sequential map kinases (MAPK: MEK, MEKK, MEKKK) and finally, ERK (Fig. 7.3). Mutations in these signal transducers render the pathway constitutively active, regardless of upstream receptor-kinase activation (see also Chap. 16). Of these proteins in melanoma, N-Ras and B-Raf are the most commonly mutated, exhibiting aberrant mutation in ~15% and over 50% of melanomas, respectively (Davies et al. 2002; Fecher et al. 2007). Further downstream, MEK mutations have also been reported, particularly in the context of acquired resistance after chemotherapy. For instance, MEK1 mutations, although at low incidence, are reported particularly following B-Raf inhibitor therapy, confer chemotherapeutic resistance MEK1 mutation (Emery et al. 2009; Murugan et al. 2009).

While mutations in N-Ras mostly reside at amino acid residue 61, deregulated B-Raf in melanoma is attributed to mutations within several hotspots, most prominently at V600E, resulting in a constitutively active kinase (Wan et al. 2004). The degree of kinase activity elicited from mutant B-Raf was calculated to be ~10 fold of its wild type counterpart (Brummer et al. 2006). Notably, while mutant B-Raf and N-Ras have overlapping downstream effectors, with ERK being the most prominent one, they also induce distinct downstream components. As illustrated in Fig. 7.3, B-Raf activity affects also Rsk, and downstream MEK-ERK kinases. These kinases in turn are shown to suppress the activity of LKB1-AMPK signaling pathway, promoting melanoma proliferation (Esteve-Puig et al.

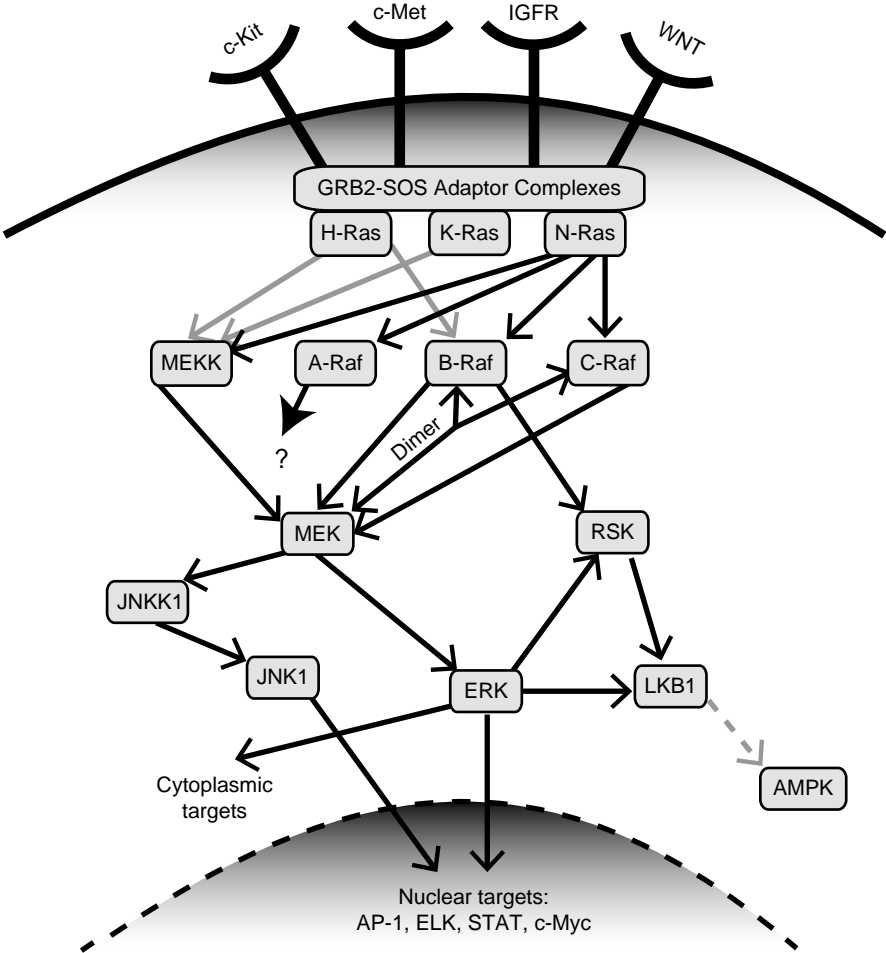


Fig. 7.3 Growth factor receptors and MAPK signaling. Membrane-bound growth factor receptors (c-Kit, c-Met, IGFR, WNT) transduce inward signaling cues upon ligand binding to their extracellular ligand-binding regions. Generally, signals are transduced through adaptor complexes containing proteins such as GRB-SOS, which recruit Ras-family members H-Ras, K-Ras, or N-Ras for activation. In melanoma, N-Ras is most commonly mutated and plays a predominant role in the activation of downstream effector kinases, Raf or MEKK. The Raf family of effector kinases consists of three main members, A-, B-, and C-Raf. While downstream signaling through A-Raf remains less studied, in melanoma B-Raf-, and C-Raf-mediated signaling predominate, and in addition to MEKK, activate downstream MEK or RSK. MEK activation results in further downstream activation of JNKK1-JNK1, as well as ERK and their subsequent cytoplasmic or nuclear transcriptional targets. Together, B-Raf and ERK can activate RSK to suppress LKB1, which functions to activate AMPK. In melanoma, this arm of AMPK activation that would normally regulate cell growth and survival is downregulated (*grey dashed arrow*). Branches of the MAPK pathway that are upregulated in melanoma appear in *black*

2009; Zheng et al. 2009). As a result, B-Raf and N-ras mutants also have distinct characteristics associated with melanoma development and progression. As noted above, the most commonly affected downstream kinase family is ERK, which is subject to constitutive activation.

Constitutive or super-activation of ERK, of the nature observed in melanoma, perturbs critical regulators of cellular behavior. B-Raf^{V600E}, a constitutively active mutant of B-Raf in many melanoma, has been demonstrated to antagonize apoptosis, for instance, by causing the ERK-dependent inhibition of apoptotic proteins, Bad, Bim, PUMA, and upregulation of anti-apoptotic proteins, such as Mcl-1 (Jiang et al. 2008; Sheridan et al. 2008; Wang et al. 2007b). Enhanced ERK activation also alters cell cycle control and proliferation, by suppressing negative cell cycle regulator, p27/Kip1 (Kortylewski et al. 2001), and importantly, by modulating isoform-specific expression of master melanocyte proliferation regulator, melanocyte-microphthalmia-associated transcription factor (M-MITF), and inducing the expression of M-MITF 6a isoform (Primot et al. 2010; Wellbrock et al. 2008). MAPK/ERK activation can further enhance proliferative capacity by promoting upregulation of other specific transcription factors including c-Jun or Brn-2, either by increasing their stability or increasing their expression, respectively (Goodall et al. 2004a; Goodall et al. 2004b; Lopez-Bergami et al. 2007). Mutant B-Raf-mediated ERK signaling also impinges on invasive cellular behaviors, conferred by altered growth or invasion regulatory proteins, such as Plexin B or matrix metalloproteinase-1 (Argast et al. 2009; Huntington et al. 2004). In fact, activation of the Ras-Raf-MAPK-Erk pathways has even been implicated in immune evasion by playing a role in the production of secreted immunosuppressive cytokines, such as interleukin-6, -10 (IL-6, -10) and VEGF by melanoma cells (Sumimoto et al. 2006).

Constitutive upregulation of ERK signaling results in rewired signaling pathways, a common occurrence in tumors, including melanoma. For example, rewired ERK signaling results in constitutive activation of c-Jun, through complementing pathways. Through upregulation of Rsk-CREB, ERK causes increased c-Jun transcription, whereas through its inactivating phosphorylation of GSK3 β , it inhibits phosphorylation of c-Jun on aa 243, which is required for its targeting for ubiquitination and degradation by FBW7 (Nateri et al. 2004; Wei et al. 2005), thereby causing increased c-Jun stability (Lopez-Bergami et al. 2007). In turn, c-Jun induces transcription of a large set of downstream targets, including cell cycle regulators, with cyclin D being one example, but also, components of additional signaling pathways. Among the latter is RACK1, an adaptor for PKC, which potentiates PKC. As a result, RACK1-PKC increases JNK activity and further activation of its substrates, including c-Jun, enforcing a feed forward signaling pathway. Among c-Jun transcriptional targets implicated in melanoma development is the upstream AKT kinase, PDK1. Jun increases PDK1 transcription, resulting in increased PDK1 activity, which feeds into the AKT and PKC pathways. Inhibition of c-Jun effectively attenuates melanoma development in xenograft model, which can be rescued by reexpression of PDK1 (Lopez-Bergami et al. 2010). As such, the ERK signaling causes activation of the JNK, PKC, PDK1, and AKT pathways—a paradigm for rewired signaling.

Constitutive activation of MAPK signaling itself has been shown to be sufficient for transformation of immortalized melanocytes, by enhancing angiogenic and invasive behavior via upregulation of VEGF and MMP-2 (Govindarajan et al. 2003). However,

synergistic intersection between upregulated MAPK signaling and other major signaling axes, such as PI3K/Akt/mTOR, can further promote other tumorigenic behavior such as the enhanced proliferation observed in uveal melanoma (Babchia et al. 2010).

Upregulated or constitutive activation of the MAPK signaling cascade correlates with poor clinical outcome (Houben et al. 2004), and is commonly attributed to activating mutations at various branch points of the pathway. At the top of the pathway are alterations in the Ras G proteins. While H-Ras and K-Ras mutations appear to correlate with benign Spitz nevi and primary lesions, N-Ras appears most frequently mutated in primary and metastatic melanoma, and is characteristic of chronically sun-exposed lesions (Ball et al. 1994; Jafari et al. 1995; Jiveskog et al. 1998; Shukla et al. 1989; van Dijk et al. 2005; van Elsas et al. 1995). Immediately downstream of the Ras proteins are the Raf kinases, and of those, B-Raf is the most frequently mutated of Raf isoforms in melanoma. B-Raf is mutated most commonly at V600E in melanoma and plays a more apparent oncogenic role than A-Raf and C-Raf, likely due to its relatively higher kinase activity (Emuss et al. 2005; Lee et al. 2005). Although B-Raf germline mutations are reported, they are not common in familial melanoma (Lang et al. 2003), suggesting that B-Raf mutations occur during melanoma development. Interestingly, mutant B-Raf^{V600E} appears to be mutually exclusive of N-Ras mutations, a phenomenon that may correlate with different melanoma tumor types and sites of origin. Consistently, unlike N-Ras mutations, B-Raf does not appear to correlate, for instance, with degree of sun-exposure (Davies et al. 2002). Interestingly, N-Ras mutation causes a switch in the usage of Raf isoforms, signaling via C-Raf (Dumaz et al. 2006).

Notably, B-Raf mutations are found in nonmalignant lesions, including congenital naevi, where mutant B-Raf is associated with upregulation of senescence markers, such as senescence-associated β -galactosidase (SA- β -gal) and a mosaic of p16INK4a (Michaloglou et al. 2005). Indeed, mutations of N-Ras and B-Raf^{V600E} alone can promote cellular senescence *in vitro*, illustrating examples of oncogene-induced senescence and highlighting the necessity for other oncogenic events to sufficiently drive tumor progression, and moreover, implicating the involvement of other contributing oncogenic events (see also Chap. 11). However, ERK activity does not always correlate with B-Raf activation, a phenomenon that is likely attributed to the functional status of ERK phosphatases. It is assumed that some ERK phosphatases are no longer active to the degree seen in nonmalignant cells, although the precise nature of the phosphatase and its deregulation are yet to be disclosed. With that, B-Raf mutation is insufficient to transform melanocytes, an event that requires secondary mutations that confer uncontrolled cell cycle progression. The notion that B-Raf cooperatively contributes to melanoma development is supported by observations that mutant B-Raf promotes nevi development in zebrafish models, and that the additive combination with further oncogenic modifications, such as loss of p53, can promote progression to invasive lesions (Patton et al. 2005). Other secondary mutations can include p16 or p19, to name a few (see Sect. 7.4). Although the role that p16INK4A may or may not play in B-Raf-driven senescence remains unclear, loss of p16INK4a can facilitate melanoma tumor formation driven by mutant Ras (Ackermann et al. 2005; Chin et al. 1997). Interestingly, a recent study shows a cooperative stabilization of β -catenin that results in silencing of p16INK4A, together with mutant N-Ras, suffice to promote melanoma progression (Delmas et al. 2007). Apart from p16INK4a, other signaling mechanisms contributing to early oncogene-induced senescence and earlier barriers to melanoma

progression continue to be identified. For example, an early oncogene-induced activation of ER stress-activated unfolded protein response was found to halt tumorigenesis, independent of conventional senescence mechanisms (Denoyelle et al. 2006).

Although epigenetic perturbations promoting the activated state of these pathways remain largely obscure, mutational status of melanoma tumors correlate with some characteristic epigenetic profiles. Melanoma tumors with B-Raf mutations, for instance, exhibit characteristic microRNA profiles. For example where specific miRNAs, such as miR-193a, -338, and -565 are downregulated, miR-191 is upregulated (Caramuta et al. 2010). And yet, greater complexity to these regulatory pathways is offered by the finding pseudogene transcripts can acting as false microRNA “decoy” targets thereby absorb and nullify the function of miRNA’s that would otherwise target select endogenous transcripts (Chen 2010; Poliseno et al. 2010). While demonstrated for PTEN, such regulation may effectively circumvent other genes that are central in control of melanoma development. As growing efforts are devoted to the sequencing of the melanoma genome, one would expect the identification of a wealth of pseudogenes that contribute to different stages of melanoma biology (Pleasant et al. 2010).

7.3 PTEN-PI3K-AKT

The phosphoinositol-3-kinase – Akt (PI3K-Akt) pathway is likely to be most frequently deregulated in melanoma (Inoue-Narita et al. 2008; Robertson 2005), although the mechanisms underlying the inactivation in most cases remains largely elusive. The PI3K pathway is an effector signaling pathway that is also positioned downstream from the aforementioned membrane receptors, including c-Met and IGFR1. PI3K transduces signal downstream from activated membrane receptors by converting cytoplasmic membranous phosphatidylinositol-4,5 bisphosphate (PIP2) into secondary lipid signaling molecule, phosphatidylinositol-3,4,5 bisphosphate (PIP3). PIP3 signaling then activates downstream, Akt/Protein Kinase B (PKB). Akt kinases comprise a three-member family of serine/threonine kinases: Akt1, Akt2, and Akt3. This family of kinases exhibit well-characterized pro-survival functions (Datta et al. 1999; Madhunapantula and Robertson 2009) (Fig. 7.4) among which Akt3 is likely to be most affected in melanoma.

Upregulation of Akt activity in melanoma is largely, but not solely attributed to deregulation of its negative regulator, phosphatase and tensin homolog (PTEN) (Parmiter et al. 1988). Although loss of PTEN protein is rampant in melanoma (Chudnovsky et al. 2005), its mutational status is less prevalent than its deregulation, attributing only a small portion of its deregulation to mutations. Notably, PTEN appears to be commonly mutated in melanoma cell lines, but such genetic mutations are rare in actual tumor samples, particularly those of metastatic grade (Goel et al. 2006; Pollock et al. 2002; Wu et al. 2003). These observations indicate that downregulation or loss of PTEN, are due to additional transcriptional and mostly post-translational modifications. Although not shown yet in melanoma, a change in ubiquitin ligase activity for PTEN, was proposed to take place by NEDD-4, a ligase that is upregulated in numerous cancer types including gastric and

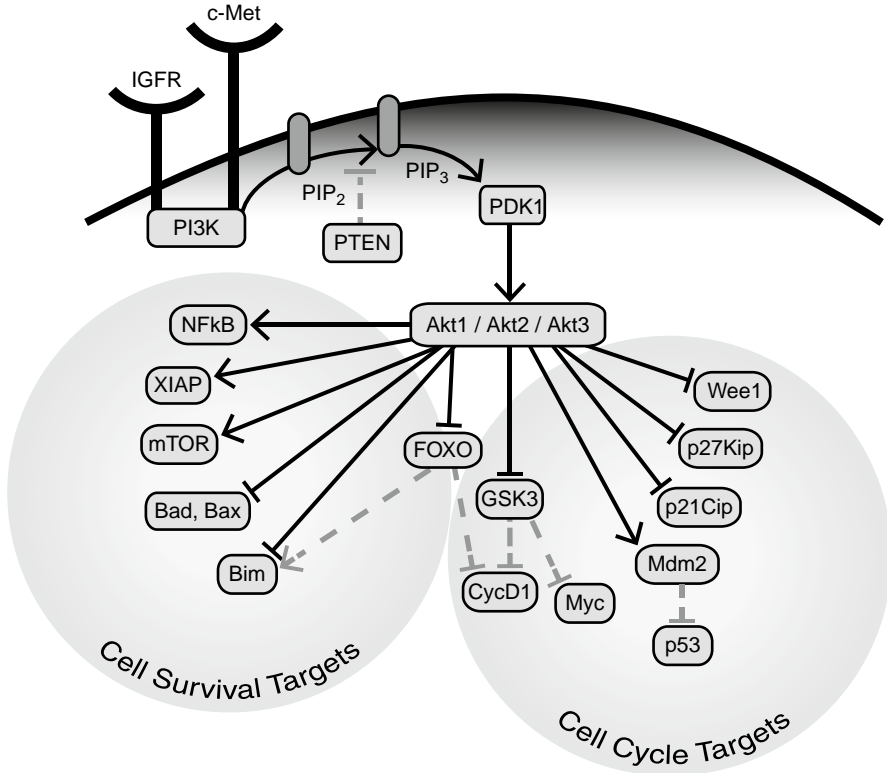


Fig. 7.4 Akt signaling. Various membrane-bound growth factor receptors (c-Met and IGFR) transduce inward signaling cues via PI3 kinase (PI3K). PI3K phosphorylates phosphatidylinositol (4, 5)-bisphosphate (PIP₂) to produce secondary messenger molecule, phosphatidylinositol (3, 4, 5)-trisphosphate (PIP₃). PIP₃ then functions to activate PDK1, which activates Akt family kinases (Akt1, Akt2, Akt3). Akt, widely recognized as a pro-survival effector kinase, generally affects cell survival by upregulating or activating numerous cell survival-related proteins, such as NFkB, XIAP, and mTor, and inhibiting cell death-related proteins, including Bad, Bax, and Bim. Akt also inhibits transcription factors, such as FOXO, which contribute to cell death or cell cycle arrest. Akt promotes cell cycle progression by inhibiting cell cycle inhibitors, including Wee1, p21Kip, p21Cip, p53 (via activation of Mdm2), and derepresses Cyclin D1 and Myc from suppression by GSK3. In melanoma, branches of the Akt pathway that are upregulated in melanoma appear in *black*; downregulated branches appear in *dashed grey*

colorectal cancers (Kim et al. 2008b; Trotman et al. 2007; Wang et al. 2007a). Another oncogenic dysregulation of PTEN stems from deregulated Fyn-related kinase (FRK, previously known as RAK). FRK, which is overexpressed in numerous cancers including melanoma, and which phosphorylates PTEN and thereby abrogates its interaction with NEDD4 (Brauer and Tyner 2009; Yim et al. 2009). Similar to PTEN loss, deregulated PIP2 processing by perturbation of inositol polyphosphate 4-phosphatase type II (INPP4B) also results in enhanced Akt signaling (Gewinner et al. 2009).

Other forms of epigenetic silencing of PTEN include promoter methylation, observed in up to 62% of patients with metastatic melanoma (Mirmohammadsadegh et al. 2006). In effect, loss of PTEN promotes an imbalanced excess of PIP3 secondary messengers to activate Akt and its downstream targets, enhancing growth and increasing survival of melanoma – a correlation inverse to that of patient clinical outcome (Dai et al. 2005).

In addition to loss of PTEN, direct changes to Akt family members also modulate the PI3K pathway in melanoma. Of the three Akt isoforms, Akt3 exhibits specific and significant upregulation in sporadic melanoma tumors, particularly those of metastatic grade (Robertson 2005; Stahl et al. 2004). This upregulation is attributed predominantly to direct increase in genomic copy numbers of the *AKT3* gene, although a recent study reports a novel activating mutation of AKT3 (E17K) found in some melanoma cases (Davies et al. 2008). Targeted siRNA-mediated silencing of AKT3 is sufficient to suppress melanoma progression and induce cell death, demonstrating the oncogenic abilities of Akt3 activation.

Akt/PKB signaling alters cell cycle dynamics through regulation of G1-S phase regulator, cyclin D3 (Spofford et al. 2006), and can moreover, affect cellular growth, metabolism, proliferation via control of VEGF expression as well as interplay with mTOR and the TORC1 and TORC2 complexes (Bhaskar and Hay 2007; Govindarajan et al. 2007; Levine et al. 2006). Akt/PKB also contributes to invasive capacity by regulating matrix metalloproteinases-2 and -9 by NF- κ B-mediated mechanisms (Kim et al. 2001). Furthermore, apoptotic mechanisms can be suppressed by Akt/PKB, by inhibiting the expression of proapoptotic proteins such as Bad or Caspase-9 (Cardone et al. 1998; Datta et al. 1997). These processes can effectively be attenuated or suppressed by the antagonistic function of PTEN, which depletes PIP3 levels via dephosphorylation. Importantly in melanoma, deregulation of the ERK-c-Jun signaling axis results in c-Jun-mediated transcriptional upregulation of PDK1, which further enhances activation of Akt (Lopez-Bergami et al. 2010). PDK1 has also been previously demonstrated to critically contribute to tumorigenesis in Akt-independent mechanisms, for instance, via PDK1 substrate SGK3/CISK, in other cancers, including breast (Vasudevan et al. 2009).

7.4 Cell Cycle

Malignant melanoma tumors are highly proliferative cells that often exhibit genomic instability (Hazan et al. 2002; Henrique et al. 2000; Satoh et al. 2000; Soyer 1991; Steinbeck et al. 1996; Urso et al. 1992). Such aggressive proliferative state is the result of a specific expansion of transformed cells that acquire imbalanced signal transduction favoring proliferation, while deregulating normal replicative senescence and apoptotic signaling (Bennett 2008). Accordingly, the stringent cell cycle regulatory mechanisms that govern cell proliferation in normal skin tissues are frequently impaired during melanoma development. For instance, G1/S checkpoint function that restricts cell cycle progression is often lost in melanoma (Sauroja et al. 2000). Similar perturbations in signaling can be traced to specific familial mutations or epigenetic dysregulation that result in the downregulation of

tumor suppressor genes that negatively regulate the cell cycle or the upregulation of oncogenic genes that promote cell cycle progression.

Patients afflicted by familial melanoma commonly exhibit conserved mutations in chromosomal locus, 9p21 – a locus that is associated with deregulation of cell cycle control. The chromosomal 9p21 locus comprises the CDK2NA gene that codes for p16INK4A and its alternate reading frame, p14ARF (also p19ARF) – cell cycle proteins that negatively regulate cell cycle progression and contribute to senescence through its control of cell cycle-promoting proteins, such as cyclin D and E and the transcription factor E2F1 (Fig. 7.5) (Bandyopadhyay and Medrano 2000; Ranade et al. 1995). Furthermore, p16INK4A binds to and inhibits cell cycle-promoting kinase, CDK4 with concomitant

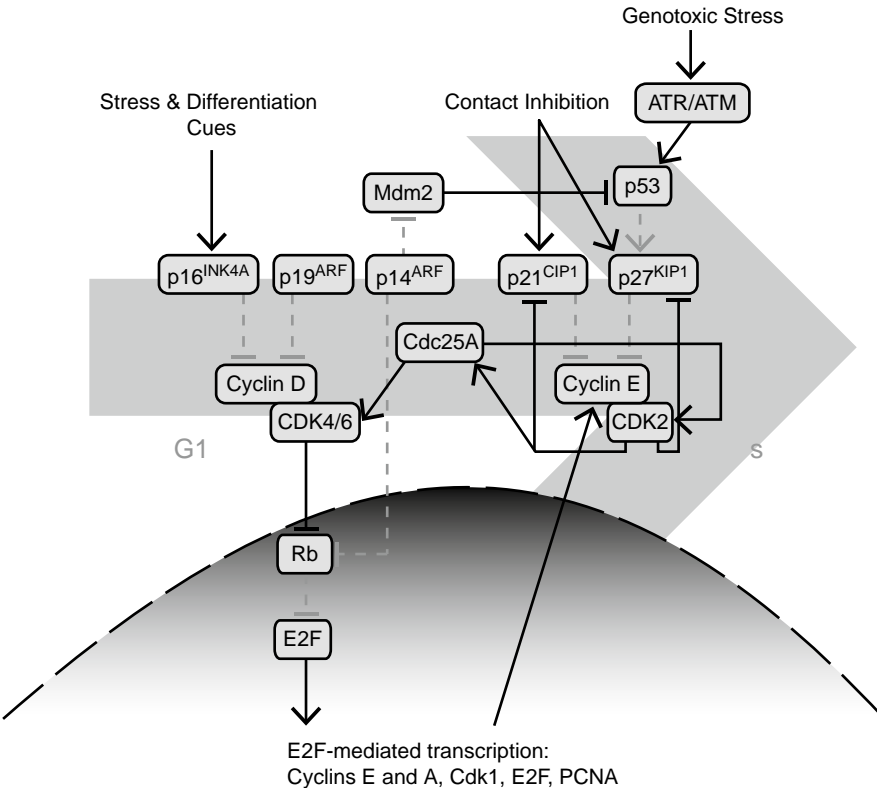


Fig. 7.5 Cell cycle and melanoma. In melanoma, loss of CDK2NA compromises major branch-points in the regulatory pathways governing progression through G1-S. Loss of p16INK4A, p19ARF, and p14ARF result in deregulation of Cyclin D/CDK4/6 that subsequently results in a loss of function of Rb and p53, promoting enhanced E2F transcriptional activity and cell cycle progression. Further, loss of function of other cell cycle inhibitors, such as p21CIP1 and p27KIP1 results in enhanced Cyclin E/CDK2 activity that positively feeds back on itself and on Cdk4/6 via Cdc25. Branches of the cell cycle regulation that are upregulated in melanoma appear in *black*; downregulated branches appear in *dashed grey*

effect on Rb, controlling E2F in the cell cycle. Germline mutations in CDK2NA were reported in melanoma and (Koh et al. 1995; Ranade et al. 1995) result in amplified CDK4-mediated signaling, and therefore perturbed control of normal cell cycle progression. Upregulation of CDK4 activity in melanoma can also result from genetic CDK4 mutations (Chudnovsky et al. 2005). These perturbations impair proper cell cycle control and the ability of melanocytes to undergo senescence (Bandyopadhyay and Medrano 2000; Haferkamp et al. 2008; Rane et al. 2002).

Notably, mutational perturbation of the alternate reading frame gene product, p14ARF, also promotes cellular proliferation. Mutation of ARF, in concert with Ras mutations, has been demonstrated to synergistically promote tumor development (Ha et al. 2007). Functional p14ARF normally contributes to p53 function by targeting and suppressing its negative regulator, Mdm2. Although mutations in p53 are relatively rare in melanoma, p53 activity can be downregulated by increased Mdm2 levels, due to mutational silencing of ARF (Freedberg et al. 2008). Of note, reports on upregulation of Mdm2 in melanoma are scarce, as are studies on reduced level or activity of p53 in melanoma (Bardeesy et al. 2001) (see also Chap. 5). Hence, the relative contribution of p53 to melanoma development remains unclear. Among possibilities that are being explored is the partial inactivation of p53 transcriptional activity whereby its ability to control cell cycle arrest or apoptotic cues are selectively impaired in melanoma.

Enhanced proliferation can also be conferred by alterations in other negative regulators of cell cycle progression, such as Rb. Notably, Rb is found to be silenced in melanoma (Yang et al. 2005), due to nonsense mutations, or as a result of inactivating phosphorylation of the translated protein (Bartkova et al. 1996; Brantley and Harbour 2000). The loss of Rb function can also contribute to the abrogation of melanocyte senescence (Haferkamp et al. 2008).

In addition to genetic mutations, altered epigenetic regulation of core cell cycle and proliferation genes also contribute to melanoma development and progression. Direct modification of chromatin structure, such as aberrant promoter hypermethylation, results in CDK2NA silencing that is reported in multiple melanoma types (Straume et al. 2002; van der Velden et al. 2001). Transcriptional repression of the CDKN2A locus can also be achieved via upregulation of repressor proteins. One example for such disruption is the overexpression of the transcriptional repressor, Id1, which transcriptionally represses CDKN2A in melanoma (Healey et al. 2010). Suppression of p16INK4A in melanoma was also shown to be mediated by β -catenin (Delmas et al. 2007).

Recent studies have highlighted the role that microRNA plays in the epigenetic control of melanoma progression (Jukic et al. 2010). For example, microRNAs that suppress proliferation are found to be downregulated during melanoma progression. Among those is miR-let-7, which targets numerous cell cycle proteins including cyclin D1/D3/A, and which is downregulated in melanoma when compared with its expression in nevi (Schultz et al. 2008). Expression of miR-34a, which is a transcriptional target of p53, is sufficient to induce G1 arrest/senescence and can act as a tumor suppressor by targeting c-Met. However, in melanoma, miR-34a is silenced by aberrant CpG promoter methylation (Lodygin et al. 2008) derepressing numerous cell cycle proteins, including RB, Cdc2, and E2F3 (Satzger et al. 2010; Yan et al. 2009). The deregulation of other microRNAs in melanoma, including miR-210 and miR-15b, has been demonstrated to promote tumorigenesis (Satzger et al. 2010; Zhang et al. 2009) (see Chap. 6).

7.5
Therapeutic Opportunities

As critical determinants of melanoma development and progression (Fig. 7.6), the individual and intersecting contributions of the MAPK-MEK-ERK and PTEN-P13K-AKT pathways have been the subject of intense scrutiny for therapeutic exploitation (Madhunapantula and Robertson 2009; Meier et al. 2005; Russo et al. 2009).

Therapeutic approaches have varied from general cell cycle inhibitory strategies, such as via inhibition of CDK2/4 (Caporali et al. 2010) or inhibition of DNA replication and cell division (Homsí et al. 2009), to the targeting of specific membrane receptors and their downstream signaling components. Indeed, application of single-agent therapies targeting membrane receptors, such as c-Kit (Woodman and Davies 2010; Woodman et al. 2009), c-Met (Abdel-Rahman et al. 2010; Kenessey et al. 2010; Puri et al. 2007a; Puri et al. 2007b), IGFR (Girnitá et al. 2006), and WNT/Frizzled (Schwartz et al. 2009) has demonstrated melanoma-inhibiting results *in vitro*, as well as in animal models. Inhibition of the downstream signaling pathways of these receptors is also a major area of exploration. Inhibition of Akt signaling *in vitro* and in mouse models show resulted in inhibited

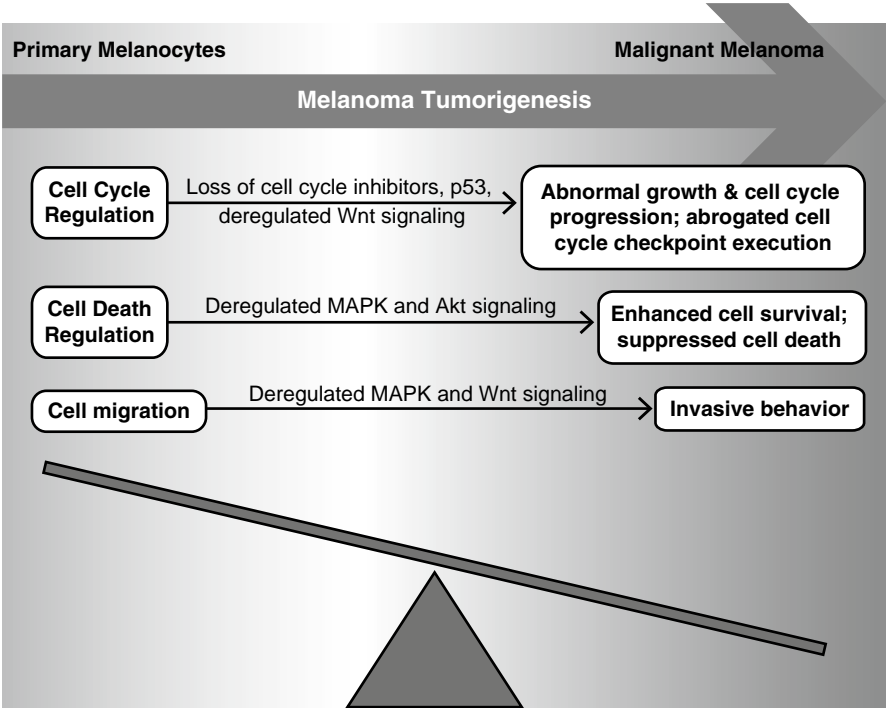


Fig. 7.6 Melanoma tumorigenesis: imbalances in cellular control. Schematic of imbalances in cellular control acquired during melanoma tumor development

melanoma proliferation, induction of apoptosis, as well as reduced tumor angiogenesis (Aziz et al. 2009; Harfouche et al. 2009). B-Raf targeting as a means of inhibiting the MAPK-MEK-ERK pathway has been a highly investigated approach, yielding controversial results that were only recently understood. Although B-Raf inhibition results in suppressed tumor growth, the inhibition is ultimately overcome by malignant cells that once again exhibit phosphorylated and active ERK. This compensation is attributed to an upregulation of activated C-Raf in the absence of B-Raf activity (Gollob et al. 2006; Hatzivassiliou et al. 2010; Kaplan et al. 2010; Montagut et al. 2008; Paraiso et al. 2010; Tsai et al. 2008; Wellbrock and Hurlstone 2010). Furthermore, recent studies show that melanoma may exhibit resistance to B-Raf-targeting therapies via upregulation of AKT3-dependent mechanisms (Shao and Aplin 2010), consistent with cooperation between mutant B-Raf and active Akt from another study showing that mutant B-Raf and PTEN loss are sufficient to generate highly invasive melanoma (Dankort et al. 2009).

However, clinical efficacy with such single-agents as first line therapy has been limited, attributed to both the initial diverse tumorigenic signaling profiles of the tumors (Smalley et al. 2009), as well as the outgrowth of subsequent tumor clones with advantageous mutations, such as MEK1 mutants that are detected subsequent to B-Raf inhibition (Emery et al. 2009). Thus numerous studies continue to explore combinatorial approaches with signaling inhibitors, such as Sorafenib (multi-tyrosine kinase inhibitor) plus carboplatin and paclitaxel (Hauschild et al. 2009), or combined inhibition of MEK and Cdk4 (Li et al. 2010).

The development of inhibitors to novel targets is actively underway and expected to complement existing therapeutic options for melanoma. Novel targets that are modified in cancer (elevated expression and/or activity) are for instance ubiquitin ligases, which provide new challenges for inhibitor design, given the requirement to interfere with protein-protein interactions. Among such ubiquitin ligases is the RING finger E3 ligase Siah2. Inhibition of Siah2 effectively blocks melanoma but also prostate, mammary, lung, and pancreatic tumors (House et al. 2009; Nakayama et al. 2009). Structure based drug design combined with high throughput screen is currently used to identify such inhibitors (Qi et al. 2010). A proof of concept screen was recently successfully completed for Siah2 inhibitors (Shah et al. 2009). Among additional targets is PDK1, which control both AKT and PKC pathways (Feldman et al. 2005; Lopez-Bergami et al. 2010; Lu et al. 2010).

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Abstract Dissociation of tumor cells from the primary site and invasion of the underlying connective tissue is a prerequisite for metastasis a process which requires a series of proteolytic events that modify not only the pericellular environment but also host-tumor communication.

A variety of proteolytic enzymes, produced either by the tumor cells themselves or by the “tumor-activated” stroma, has been implicated in the pathogenesis of cancer including melanoma. We shortly review the most important and well characterized proteolytic enzymes, their degradative activities, and the impact that those events have on the progression of tumor cells through tissues. We will especially concentrate on those enzymes which participate in cellular communication, cell adhesion and matrix remodeling.

8.1 Introduction

Interaction between tumor cells and the stromal compartment plays a major role in cancer progression. Such interactions are particularly important while tumor cells dissociate from the primary tumor, invade the surrounding connective tissue, and penetrate vessel walls. While tumor cells invade and degrade the neighboring stroma they generate a permissive microenvironment convenient for their growth, migration and metastatic spread.

The tumor stroma, including that of melanoma, is characterized by a complex structure and is composed by matrix proteins and a rich cellular population which includes

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fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, macrophages, and other inflammatory cells. Several molecular modifications of the microenvironment occur by the activity of tumor cells themselves or by stimulated stromal cells and include release of matrix or cell surface bound factors, bioactive matrix fragments as well as cell–cell and cell–matrix contacts (see also Chap. 14).

All these events are controlled by the activity of several proteolytic enzymes produced by either tumor or stromal cells (Fig. 8.1). Proteases involved in these processes are classified into serine-, cysteine-, aspartyl-, and metalloproteinases according to structural characteristics of their active enzymatic center. In this review we will give an overview on those enzymes that have been implicated in the development of malignant melanoma and their role in the pathogenesis of this disease.

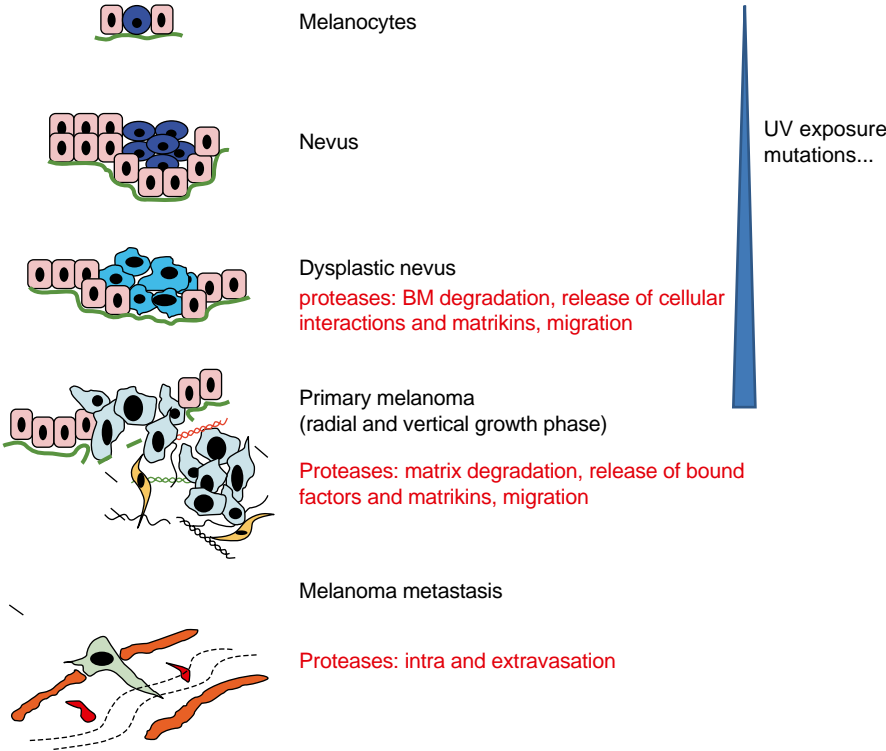


Fig. 8.1 Transformation of nevus to melanoma. Benign transformation of melanocytes following accumulation of mutations (e.g., upon UV exposure), of a nevus which progress into dysplastic nevi. The following step into a malignant radial and vertical growth melanoma is characterized by the activity of several proteolytic enzymes. Proteolysis mediates cellular functions leading to malignant cell progression through the tissue and contributes to the intravasation of melanoma cells into blood and lymph vessels

8.2 MMPs

In normal skin matrix remodeling occurs by a slow but continuous synthesis and constitutive degradation and reconstruction of matrix components. MMPs are strongly involved in perpetuating skin integrity in general. Controlled degradation of connective tissue is necessary during wound repair, regulation of tissue architecture, and embryonic development (reviewed in (Page-McCaw et al. 2007)).

Increased expression of MMPs has been observed in different malignancies and has been implicated in the enhanced ECM degradation. On the contrary, altered ECM deposition and degradation resulting from modifications of TIMPs and collagenase expression such as during aging, may lead to a higher cancer incidence (Anisimov 2001; Reed et al. 2000) (see also Chap. 11). In melanoma, MMPs were mainly located at the tumor-stroma border and are produced by either tumor cells or by stromal cells activated by tumor-derived soluble factors. In addition, growth factors and cytokines secreted by tumor-infiltrating inflammation also modulate MMP expression in melanoma and stroma cells (Coussens et al. 2002). *In vitro* and *in vivo* analysis have indicated an important role of the matrix itself for the induction of MMPs such as MMP-2/-9, and MMP-14 by invasive melanoma (Baumann et al. 2000; Hofmann et al. 2000; Kurschat et al. 1999). In human melanoma tissues, proteolytic activities of MMP-2/-9 were localized in peritumoral areas, while no activity was observed within the tumor nodules (Kurschat et al. 2002). Expression of MMP-14 was instead mainly localized in melanoma cells located at the invasive front of the tumor (Hofmann et al. 2000; Kurschat et al. 2002). Thus localization of both MMP-2 and MMP-14 at the interface between tumor and stroma may be required for enzyme activation and matrix degradation and highlight how the different cells cooperatively act to promote tumor progression.

Expression of the interstitial collagenases MMP-1 and -13 which are key enzymes in the cleavage of fibrillar collagens is detected during invasive vertical growth phase in melanoma cells thus indicating that this is a late event in melanocytic tumor progression (Airola et al. 1999). However, even though the studies from Airola et al. (1999) show expression of both collagenolytic enzymes in tumor cells *in vivo*, several other *in vivo* and *in vitro* studies have indicated production of these enzymes primarily by peritumoral fibroblasts (Wandel 2000; Löffek et al. 2005; Uriá et al. 1997; Zigrino et al. 2009). We have recently shown that stromal expression of MMP-13 plays an important role in tumor growth of melanoma. Melanoma cells injected into the skin of mice with host-derived, inactivated MMP-13 formed smaller tumors and developed fewer metastases as compared to MMP-13 producing wild type animals (Zigrino et al. 2009). Reduced tumor growth and metastasis is likely to be caused by the lack of MMP-13 which we could show in wild type animals being strongly up-regulated in fibroblast-like cells of the adjacent stroma. In addition, we and others could also show that MMP-13 is important in tumor vascularization (Lederle et al. 2010).

Beside MMP-13 several other MMPs have been implicated in both pro- and anti-angiogenic processes during tumor development. Recent studies have identified a novel mechanism by which MMP-1 promotes angiogenesis. MMP-1 proteolytically activates

protease activated receptor-1 (PAR1), a thrombin receptor that is highly expressed in endothelial cells (Blackburn and Brinckerhoff 2008). In addition, MMP-14 is believed to facilitate endothelial cell migration and tube formation processes *in vitro* and *in vivo* (Chun et al. 2004; Mimura et al. 2009). An anti-angiogenic activity of MMPs has been instead shown by the activity of MMP-12 and MMP-9 that hydrolyze plasminogen to form the angiogenesis inhibitor angiostatin (Patterson and Sang 1997; Raza et al. 2000). The importance of MMP-14 in invasive and angiogenic processes was recently highlighted by *in vivo* studies from Devy et al. (2009) showing that inhibition of MMP-14 using a selective human MMP-14 antibody inhibited proliferation and angiogenesis of human tumors in preclinical studies. Meanwhile various endogenous angiogenesis inhibitors were identified which are released by MMPs proteolytic cleavage of plasma proteins or ECM components, such as collagen XVIII for the generation of endostatins, perlecan for that of endorepellin and collagen IV for the generation of tumstatin (Fjeldstad et al. 2005; Hamano and Kalluri 2005). Thus the balance between MMPs pro and anti-angiogenic activities and their tissues inhibitors, is likely to dictate the outcome during tumor development. These data, however, make clear that broad range MMP inhibitors cannot be useful for the treatment of patients with advanced tumor diseases.

Another important role of MMPs is the shedding of cell surface receptor such as cleavage of E-cadherin (see also Chap. 9). Shedding of these cell–cell receptors has been shown to occur by the activity of MMP-7 and MMP-3 thereby regulating the invasive capacities of transformed or injured epithelial cells (McGuire et al. 2003; Noe et al. 2001). A significant increase in serum soluble E-cadherin levels was detected in melanoma patients with advanced disease, thus suggesting that in melanoma shedding, apart from transcriptional regulation of the receptor, contributes to render melanoma cells refractory to the keratinocyte-mediated regulation (Billion et al. 2006; Hsu et al. 2000). Another example for MMP-mediated receptor shedding is that of CD44, known as receptor for hyaluronan. Cleavage of CD44 by MMP-14 results in increased melanoma cell motility *in vitro* and likely plays a role for tumor progression *in vivo* (Kajita et al. 2001; Nakamura et al. 2004). Due to its structure as a transmembrane protein MMP-14 was demonstrated to localize at specialized membrane areas such as lamellipodia. In agreement with this observation high local concentrations of active MMP-14 on the cell membrane are believed to play an important role in cellular migration of melanoma cells (Friedl and Wolf 2008).

Increased production of MMP-2 and MMP-14, and deposition of the $\gamma 2$ chain of laminin 332 and/or its cleavage fragments have been observed in melanoma cells *in vitro* (Seftor et al. 2001). This process likely contributes to tumor progression also *in vivo* where the cleaved form of laminin 332 was found in tumors and in tissues undergoing remodeling but not in quiescent tissues (Lohi 2001; Patarroyo et al. 2002).

Despite increased expression and in some cases also activity of MMPs was shown to correlate with advanced stages of tumors, other studies showed that MMPs may also possess anti-tumor activity. The neutrophil collagenase, MMP-8, is one typical example: MMP-8 ablation in mice strongly increased the incidence of skin tumors. Notably, restoring the neutrophil pool by bone marrow transplantation re-established the natural protection against tumor development in male mice (Balbin et al. 2003). In addition, in high invasive breast cancer cells, transduction with MMP-8 decreased the metastatic performance *in vitro* and *in vivo* (Montel et al. 2004). A study of Palavalli et al. (2009) has

identified 23% somatic mutations of MMPs in human melanoma and five of these were found in the MMP-8 gene associated with loss of enzymatic activity. Forced expression of the wild type gene resulted in reduced melanoma growth *in vivo* suggesting that this MMP is a tumor suppressor (Palavalli et al. 2009).

MMP-9 was also found to have tumor-inhibiting activity. In grafts of melanoma or lung carcinoma cells in either wild type or MMP-9 depleted animals demonstrated that this metalloproteinase functions as a metastasis suppressor but does not interfere with the growth of grafted tumors (Gutierrez-Fernandez et al. 2007). MMP-9 mediates the enzymatic cleavage of the basement membrane collagen type IV generating a proteolytic fragment, tumstatin, that functions as an endogenous inhibitor of pathological angiogenesis and suppresses the activity of endothelial cells as well as growth of melanomas (Hamano and Kalluri 2005). In addition, Coussens et al. (2000) have shown by reconstitution of bone marrow in MMP-9 knockout mice with bone-marrow-derived cells of MMP-9 control animals that peritumoral inflammatory cells are the stromal source of MMP-9 during epithelial carcinogenesis and are required to sustain tumor growth. Thus, to address the role of an MMP for tumor progression solely by expression studies may be insufficient, whereas addressing enzymatic activities may rule out the specific role of a proteolytic enzyme in pathological processes.

8.3 ADAMs

ADAMs (A disintegrin and metalloproteinases) are likely to contribute to the proteolytic events necessary for tumor invasion and metastasis (Bergers and Coussens 2000; Schlondorff and Blobel 1999). The domain structure of the ADAMs is highly homologous to the domain structure of hemorrhagic snake venoms, known as SVMPs (Snake Venom Metalloproteases). These proteins also contain a metalloproteinase, a disintegrin, and a cysteine-rich domain (Jia et al. 1996). The disintegrin domain of the snake venom disintegrins albolabrin and concortrostatin can interfere with the integrin-mediated adhesion of melanoma cells to basement membrane components thus blocking extravasation of tumor cells from the blood into organs and inhibiting metastasis (Beviglia et al. 1995). Also treatment with jararhagin, either with active or metalloproteinase inactivated, was able to significantly decrease the lung metastasis of melanoma xenografts (Correa et al. 2002). Based on the structure homologies between the ADAMs and the snake venom proteases one can assume that the members of the ADAM family could also be implicated in processes which modulate tumor growth and metastasis. Based on their domain structure, these proteins can exert modulating effect on both the migratory behavior of tumor cells as well as on the proteolytic processes with regard to tumor invasion. In support of this, their over-expression has been detected in a variety of tumors (reviewed in (Murphy 2008)). Extensive expression analysis has been performed on breast and pancreatic carcinomas where expression of ADAM-9, -10, -12 and -15 have been reported to be increased (Murphy 2008). However, very limited information is available on the role of the different ADAM family members in the pathogenesis of melanoma. In our own studies, we found ADAM-9 being

strongly expressed in primary melanomas and low in metastases (Zigrino et al. 2005). Presently the contribution of this protease to development, invasion, and metastasis of melanoma is unclear. Another member, ADAM-10, was also found to be regulated in melanoma. However opposite expression levels were detected with high amounts in metastases and low levels in primary tumors (Lee et al. 2009). Although the exact role of ADAM-10 in tumorigenesis is not understood, the very recent identification of ADAM-10 mediated constitutive shedding of CD44 in human melanoma cells favors the idea that this protease facilitates melanoma cell proliferation (Anderegge et al. 2009).

Horiuchi et al. (2003) have shown that ADAM-15 expression whereas not required for developmental angiogenesis or for adult homeostasis, may be necessary for mouse melanoma growth. Using mouse carrying a complete depletion of ADAM-15, these authors could detect consistently smaller tumors developing after injection of B16F0 melanoma cells in ADAM-15 knockout animals as compared to correlative controls, but not molecular mechanism for this effect has been shown. ADAM-15 may therefore represent a novel target for the design of inhibitors of pathological neovascularization.

8.3.1

ADAMs as Sheddases

A dual function has been recently attributed to ADAM-8 being both a negative regulator of retinal vascularization and growth of heterotopically injected B16F0 melanoma cells (Guaiquil et al. 2010) by modulating processing of several molecules involved in angiogenic processes such as CD31, EphB4, and EphrinB2. Inactivation of ADAM-17 in endothelial cells significantly reduced pathological neovascularization in a mouse model for retinopathy of prematurity, and affected the growth of heterotopically injected murine melanoma cells. Surprisingly, differences in tumor growth could not be attributed to altered vascularization as no differences in CD31 positive tumor vessels were detected but hypothetically to the generation of factors from endothelial cells that contribute to tumor growth (Weskamp et al. 2010). All these data come, however, from mouse models (see also Chap. 15) and whether these events may also be affected by these ADAMs during development of human melanoma is not yet clear.

Processes mediated by ADAM proteases may be relevant for melanoma growth and invasion and these include shedding of ectodomains such as the epidermal growth factors. By shedding of the ectodomains, soluble, diffusible factors are released, which can carry out different biological functions as compared to their membrane-anchored protein form. The EGF pathway is active in various cancer cell types and sustains their survival, proliferation, and motility. Among these ligands and receptors, TGF- α , HB-EGF, EGFR and HER2 have pivotal roles in pancreas, colon, breast and lung cancers, as well as melanoma (Blöbel 2005). ADAM-17 is an excellent example, which releases the cytokine TNF- α from its inactive membrane-anchored form (Black and White 1998). One other shedding event relevant for cancer development is that of the ligands of the activating natural killer cell receptor, NKG2D, MHC class I chain-related molecule A and B (MICA and MICB). This shedding has been studied *in vitro* in cervix cancer cells and is thought to be one of the mechanisms that promote resistance to the

immunosurveillance by NK cells (Waldhauer et al. 2008). In other cancer cells, such as hepatocellular carcinoma, ADAM-9 has been shown to be responsible for the shedding of MICA and treatment with anticancer drug Sorafenib, causing decreased expression of ADAM-9 was followed by increased MICA expression of the cell surface (Kohga et al. 2010). However, recently shedding of the NKG2D ligand MICA was also shown to be mediated by MMP-14 and to occur independently from ADAMs (Liu et al. 2010). Which protease is responsible for the shedding of these NKG2D ligands is likely the resultant of the expression profile in the tumor cell type involved, but this is hypothetical and not yet clearly investigated. One can envision that in addition to the protease expression profile of a specific tumor also the tumor microenvironment may dictate which enzyme performs the required shedding. Because most human cancer cells express both MICA and MICB it is likely that a similar event takes place in melanoma. Together with the evidence that expression of shedding-resistant forms of MICAs prevents mouse prostate tumor growth *in vivo* (Wu et al. 2009), shedding of MICAs may represent an important target for cancer immune therapy.

Shedding of surface receptors mediating cellular interactions has also been demonstrated for ADAM proteases. E-, N-, and VE-cadherins have been shown to be substrates for ADAM-10 (Reiss and Saftig 2009) and their shedding resulted in profound alterations in cellular interactions, migration and cell proliferation. ADAM-10-mediated L1 release is involved in the motility and invasion of lymphoma, lung carcinoma and melanoma cells (Gutwein et al. 2000; Lee et al. 2010).

8.3.2

ADAMs Mediate Cellular Interactions

Apart from modulating cell adhesion by shedding events, one important aspect of ADAMs, is their direct involvement in mediating adhesive events through their adhesive domains. *In vitro* studies have shown that ADAM-15 interacts with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins (Nath et al. 1999), whereas ADAM-2 binds to $\alpha_6\beta_1$ integrin and ADAM-9 to $\alpha_6\beta_1$, $\alpha_v\beta_5$, and $\alpha_3\beta_1$ integrins (Almeida et al. 1995; Nath et al. 2000; Zhou et al. 2001). The interactions of ADAM proteases with cellular receptors have been proven to be of major importance in cell adhesion and fusion processes as for instance during spermatogenesis and myo- and osteogenesis (Edwards et al. 2008). The secretion of a soluble splice variant of ADAM-9 by activated stromal cells is known to induce colon carcinoma cell invasion *in vitro* through binding to $\alpha_6\beta_4$ and $\alpha_2\beta_1$ integrins (Mazzocca et al. 2005).

8.4

ADAMTS

Another class of ADAM-related enzymes is the ADAMTS. These proteins have additional thrombospondin domains which are believed to function as a sulfated glycosaminoglycan-binding domain (Tang 2001). Silver et al. (2008) have recently shown that ADAMTS-20

induced cleavage of versican results in modulation of Kit signaling during melanoblast colonization of the skin (Silver et al. 2008). Indeed, both, versican and Kit have been found highly expressed in melanoma and correlated with disease progression (Domenzain et al. 2003; Giehl et al. 2007). High levels of ADAMTS transcripts have also been detected in tumor biopsies and cell lines from osteosarcoma and melanoma (Cal et al. 2002). Expression and activity of ADAMTS proteins has not yet carefully been analyzed in melanoma but given the various functions attributed to these enzymes in development and other diseases, it is conceivable that these enzymes are also expressed and involved in melanoma development.

8.5

Cathepsins

Cathepsins described in melanomas are both cysteine (B, H, K, and L) and aspartyl proteases (D and E). These enzymes are predominantly intracellularly localized within the lysosomal compartment. Some of these enzymes can be associated with the plasma membrane of melanoma cells *in vitro* or their secretion from lysosomes can be induced by cell contact with collagen type I (Klose et al. 2006; Moin et al. 1998). Frohlich et al. (2001) have analyzed expression of cathepsins in normal skin, nevi, and melanoma samples to obtain information about their role and their regulation in melanoma. Activities and expression of the cathepsins B and L were found to be increased in all melanocytic lesions (Frohlich et al. 2001). Interestingly, Cathepsin H expression correlated inversely with the invasive potential of the lesion (Frohlich et al. 2001). The aspartyl protease cathepsin D has been detected in tumor cells as well as in the peritumoral stroma in melanoma (Kageshita et al. 1995). Expression of Cathepsin D has also been inversely correlated with melanoma such that its expression is down-regulated in melanoma cells as compared to melanocytes, thus suggesting a role for this enzyme in cellular transformation (Bernard et al. 2003). *In vitro*, cathepsin B activity was shown to be necessary for invasion of dermal connective tissue by high invasive melanoma cells (Dennhofer et al. 2003). Furthermore, forced expression of Cathepsin L in human melanoma cells increased their metastatic potential thus increasing their tumorigenicity (Jean et al. 1996). Neutralization of cathepsin L *in vivo* by lentiviral transduction of melanoma cells with the anti-cathepsin L ScFv fusion protein resulted in inhibition of the tumorigenic and metastatic phenotype of human melanoma (Rousselet et al. 2004). However, a recent work from Matarrese et al. (2010) could show that Cathepsin B but not L and D inhibition by several targeting approaches could efficiently reduce tumor growth and metastatic potential of human melanoma cells *in vitro* and *in vivo* (Matarrese et al. 2010).

Another recently described cysteine protease expressed in melanoma is Cathepsin K. This enzyme has been extensively studied during bone resorption processes where it mediates degradation of various collagens (Bossard et al. 1996). Whereas Cathepsin K is not expressed in normal skin, its expression has been localized in skin fibroblast during scar formation (Runger et al. 2007). Cathepsin K has been detected in melanocytes and nevi

and its expression is increased in human primary cutaneous melanoma and metastasis (Quintanilla-Dieck et al. 2008). Expression of Cathepsin K was also detected in melanoma cells where it may be implicated in collagen type IV intracellular degradation (Quintanilla-Dieck et al. 2008).

8.6

Serine Proteases

Among the group of serine proteases involved in extracellular matrix degradation, plasmin, plasminogen activators, thrombin, Cathepsin G, and elastases are of particular interest.

The serine protease plasmin has been shown to activate most proMMPs *in vitro* (Murphy et al. 1999). However, it has not been proven whether the function as proMMP activator is also fulfilled *in vivo*. Their proteolytic activity has also been shown toward extracellular matrix components and cell surface receptors thereby influencing the tumor microenvironment in multiple ways (Andreasen et al. 1997).

Release of elastin fragments by the serine protease elastase was shown to enhance melanoma cell invasion through type I collagen and increase angiogenesis by the induction of MMP-14 expression (Hornebeck et al. 2005). One hypothesis advanced by Antonicelli et al. (2007) is that these peptides could catalyze the vertical growth phase transition in melanoma through increased expression of MMP-2 and MMP-14 (Antonicelli et al. 2007). Plasminogen activation by the urokinase type (uPA) or the tissue type plasminogen activators (tPA) generates plasmin whose expression is associated with ECM degradation (Li and Wun 1998). Generated plasmin can also excise the angiostatin fragment from plasminogen thus regulating angiogenic processes. uPA is bound to the surface of tumor cells by means of a specific receptor (uPAR) and engagement of this receptor can act as a survival factor for melanoma by downregulating p53 thus inducing an anti-apoptotic effect (Besch et al. 2007). uPA expression has been correlated to the ability of blue nevi to transform, as gene ablation in mice inhibited melanoma formation (Shapiro et al. 1996). A synergism between the uPA/uPAR system and MMP-9 was shown to mediate the IFN- γ and TNF- α induced invasive phenotype of murine melanoma cells (Bianchini et al. 2006). Increased uPA and MMP-2/-9 expression, accompanied by down-regulation of E-cadherin was observed following overexpression of PKC- γ in mammary cells leading to acquired invasive abilities of those cells which became tumorigenic and able to spontaneously metastasize (Mazzoni et al. 2003). Overexpression of Hsp27 in the melanoma line A375 leads to reduced malignant phenotype likely by increased expression of E-cadherin and of both, uPA and PAI-1, its inhibitor (Aldrian et al. 2003). It is known that once the uPA/PAI-1 complex is formed, it can be degraded in the lysosomes thus either the increased protease/inhibitors are cleared by degradation or even if free in the environment activity of uPA may be hampered by the concomitant production of inhibitors (Ossowski and Aguirre-Ghiso 2000).

Human melanoma cells produce tPA which is bound to the cell surface (De Vries et al. 1996). *In vivo*, high tPA expression has been correlated with good prognosis (Ferrier et al.

2000). In these studies, lesions with more than 51% tPA-positive tumor cells were found to have the best prognosis, as compared to those with lower expression. Further, taking together tPA positivity, Breslow thickness, microscopic ulceration, and sex, showed that the extent of tPA tumor cell positivity was an independent prognostic factor for distant metastasis-free interval and for the duration of survival (Ferrier et al. 2000). A potential reason for the better outcome is the generation, upon activation of plasmin and thereby cleavage of plasminogen, of angiostatin which may inhibit tumor-induced angiogenic processes.

Another serine protease which has also been investigated in melanoma is thrombin. Thrombin cleaves the N-terminus of protease-activated receptor-1 (PAR-1) that binds the receptor and activates signaling via G-proteins (Shapiro et al. 2000). PAR-1 can also be activated by ligands other than thrombin such as trypsin and plasmin (Kawabata and Kuroda 2000). PAR-1 is overexpressed predominantly in melanoma primary tumors and in metastatic lesions as compared with common melanocytic nevi (Massi et al. 2005). The importance of activating this pathway is shown in a recent report from Melnikova et al. (2009). The authors showed that PAR-1 activation leads to expression of melanoma cell adhesion molecule MCAM/MUC18 (MUC18) thereby mediating melanoma cell adhesion to microvascular endothelial cells, transendothelial migration, and ultimately, lung metastasis retention (Melnikova et al. 2009).

8.7

Summary

Although fundamental knowledge about the molecular processes of tumor progression is increased in recent years, their clinical applications are limited.

Concerns remain about the consequences of inhibiting the biological function of cell proteolytic enzymes. Several reports have contributed to increase the knowledge on the regulation of proteases and their substrates and highlighted that this process is complex and multifactorial. Experimental evidence strongly suggests that proteases of the same class may display a dual function depending on the cellular source and act both as a tumor-promoter and a tumor-suppressor. Thus, for a more specific targeting, a deeper knowledge of the proteolytic functions is needed and promises come from the field of degradomics which started to uncover new proteases and physiological substrates, and identified new and known regulatory pathways that are controlled by proteolytic processing.

The direct targeting of these proteases in a cell specific manner with, for example, monoclonal antibodies or inactivating peptides, or the inhibition of the activity of their processed substrates may, therefore, be developed as potentially useful therapeutic strategies.

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Cell–Cell and Cell–Matrix Contacts in Melanoma and the Tumor Microenvironment

9

Silke Kuphal and Nikolas K. Haass

Abstract Cell-contacts are essential for intercellular communication and are involved in proliferation, differentiation, and homeostasis. Melanocytes establish multiple contacts with keratinocytes, which in turn control melanocyte growth and expression of cell surface receptors. Most melanomas arise within the epidermis (melanoma in situ) and then invade across the basement membrane. These melanoma cells escape from control by keratinocytes through five major mechanisms: (1) down-regulation of receptors important for communication with keratinocytes such as E-cadherin, P-cadherin, desmoglein, and connexins; (2) up-regulation of receptors and signaling molecules important for interactions between melanoma cells and other melanoma cells, fibroblasts, or endothelial cells, such as N-cadherin, Mel-CAM, and zonula occludens protein-1 (ZO-1); (3) de-regulation of morphogens such as Notch receptors and their ligands; (4) loss of anchorage to the basement membrane due to altered expression of cell–matrix adhesion molecules; (5) increased expression of metalloproteinases.

Melanoma depends on, interacts with and reacts to its stroma, including extracellular matrix, growth factors, cytokines, fibroblasts, endothelial cells, and immune cells. In turn, melanoma is known to produce factors that influence its environment, and may force it to alter cell–cell communication.

In the current review we describe the alterations in cell–cell contacts in melanoma and the tumor microenvironment associated with melanoma development and progression.

Abbreviations

CDH	cadherin
Cx	connexin(s)
Dsg	desmoglein
GJ	gap junction
GJIC	gap junctional intercellular communication, Dsc 1–3, desmocollin

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9.1

Adherent Junction of Cadherins

Cross-talk between benign precursor cells, malignant cells, and surrounding host cells influences tumor development. Already in 1914, Theodor Boveri recognized the importance of changes in tumor cell adhesion for the development of cancer (Boveri 1914). Among the molecules involved in this intercellular communication are cadherins, which play a critical role for the homeostasis of normal skin and also during tumor formation and progression (Fig. 9.1). The identification of cadherins in the late 1970s and early 1980s was primarily motivated by an interest in understanding the mechanisms of cell adhesion during development (Franke 2009).

Cell–cell as well as cell–matrix adhesions are critical for cells and tissues to respond to mechanical stimuli from their environment. Both cell–cell and cell–matrix adhesions bear intrinsic mechanosensitivity, which allows them to promptly respond to stress and effectively propagate signals controlling cell shape and motility. This mechanosensitive response has been associated with pronounced changes in the size and molecular composition of specific adhesion sites and, consequently, the signals evoked by those adhesion sites. In polarized epithelia of vertebrates, the adherent junction is part of the tripartite junctional complex localized at the juxtaluminal region, which comprises the tight junction (TJ, see below), adherent junction (AJ), and desmosomes (macula adherens).

More than 80 proteins belong to the cadherin superfamily and are separated into the following “adherent-junction” (AJ) subgroups in vertebrates:

1. Classical adhesive cadherins of type 1 (6 members) and type 2 (13 members), e.g., E-, N-, P-, R-, and VE-cadherin. The classical cadherin family comprises 19 members that share a common domain organization of five repetitive extracellular calcium-binding subdomains (Overduin et al. 1995). Most of these classical cell–cell adhesion molecules are connected to the actin filaments and microtubules of the cellular cytoskeleton via catenins. The four known catenins alpha-, beta-, gamma (plakoglobin)-, and delta (p120)-catenin are important regulatory elements either for the sustained cell–cell adhesion or signaling cascades into the cell.
2. The “nonclassical” desmosomal cadherins, transmembrane proteins of desmosomes, are for example desmocollin 1–3 (Dsc 1–3) and desmoglein 1–4 (Dsg 1–4). They are connected to intermediate filaments.
3. Finally, there are nonclassical cadherins, like the Protocadherins (e.g., Protocadherin 15, cadherin 23), H-cadherin, and Cadherin-like molecules (e.g., Fat, Dachshous, Flamingo, or Ret) belonging to the cadherin superfamily.

The most important classical cell–cell adhesion molecules of the skin and during melanoma development are E (epithelial)-cadherin (CDH-1), N (neuronal)-cadherin (CDH-2), and P (placental)-cadherin (CDH-3), which belong to the group of calcium-dependent glycoproteins. Certainly, this group of classical adhesion molecules can be extended with atypical VE (vascular endothelial)-cadherin (CDH-5, CD144) and the nonclassical

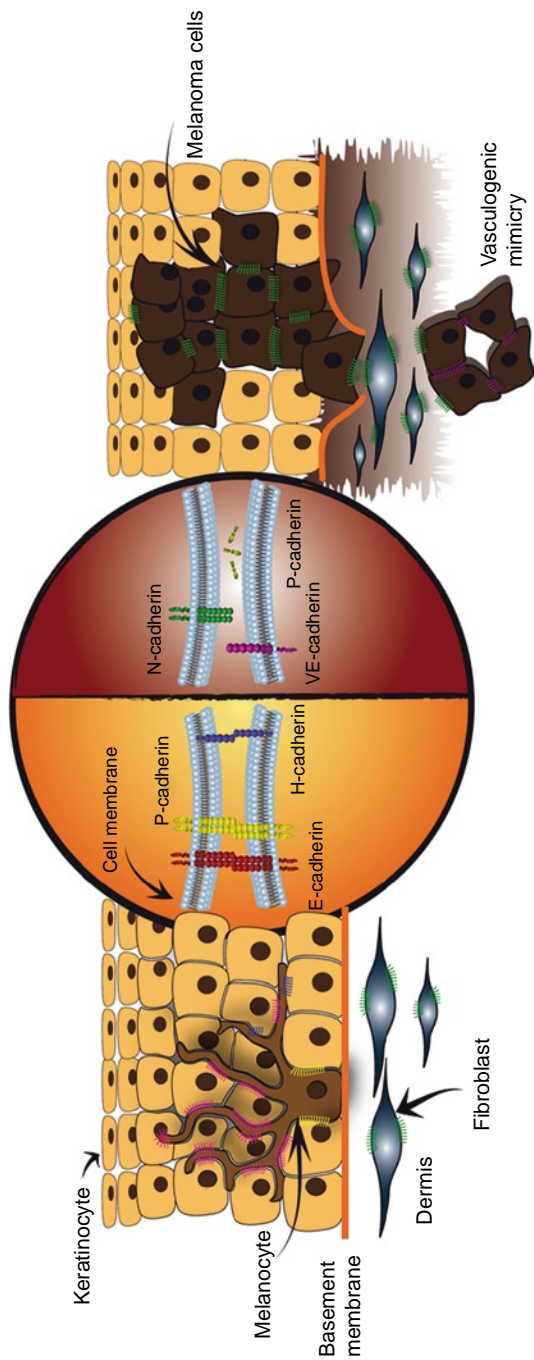


Fig. 9.1 Overview of the cadherin repertoire in skin and melanoma (Illustration R.J. Bauer)

cadherin H (heart)-cadherin (CDH-13) (Fig. 9.1). In normal epidermis, melanocytes and keratinocytes are mostly connected via E-cadherin, P-cadherin, and H-cadherin (Kuphal et al. 2009; Nishimura et al. 1999; Tang et al. 1994). Whereas melanocytes in the basal layer of the epidermis seem to contain predominantly E-cadherin and H-cadherin, those residing in hair follicles are rich in P-cadherin (Nishimura et al. 1999). In contrast, N-cadherin is expressed on fibroblasts and vascular endothelial cells of normal skin (Hsu et al. 1996).

9.1.1

Loss of E-Cadherin in Tumorigenesis

E-cadherin is the major cadherin in polarized epithelial cells. Furthermore, the crosstalk between melanocytes and keratinocytes mediated by E-cadherin plays an important role in human epidermis. The normal melanocytic phenotype and controlled proliferation of melanocytes are strictly regulated by keratinocytes via E-cadherin. The E-cadherin knock-out mouse is lethal in early embryonic stages (Larue et al. 1994) supporting the finding that E-cadherin has an essential role in morpho- and organogenesis. In skin development, there is evidence that E- and P-cadherin play some role in guiding melanocyte precursor cells to their final destination in the epidermis (Nishimura et al. 1999).

Malignant transformation of melanocytes is frequently attended by loss of E-cadherin expression and induction of N-cadherin (Hsu et al. 1996). This leads to the loss of the regulatory dominance of keratinocytes over melanocytes. The degenerated melanocytes/melanoma cells express N-cadherin to get into contact to fibroblasts and vascular endothelial cells during migration and invasion into the tumor stroma, dermis, lymph, and blood vessels (Hsu et al. 2000) (Fig. 9.2). The switch of the cadherin class is an interesting phenomenon of melanoma cells and in epithelial-mesenchymal transition (EMT) in general.

However, immunohistochemical examination of primary melanomas and their metastases has revealed that a proportion of melanoma cells are still E-cadherin positive and present little, if any, N-cadherin (Danen et al. 1996; Hsu et al. 1996; Sanders et al. 1999; Silye et al. 1998). Therefore, the cadherin switch as an obligatory prerequisite of malignant behavior is still controversial and might depend on the subtype of the melanoma examined. However, immunohistochemistry data could not show whether the expressed E-cadherin is really functionally active regarding adhesion or still possesses signaling function. The general consensus is that E-cadherin is a tumor invasion suppressor.

9.1.1.1

Regulators of E-Cadherin

The mechanism by which E-cadherin expression is lost during malignancy differs between tumor entities. Loss of E-cadherin function can be caused by various genetic or epigenetic mechanisms. In patients with diffuse gastric cancer and breast cancer, the E-cadherin

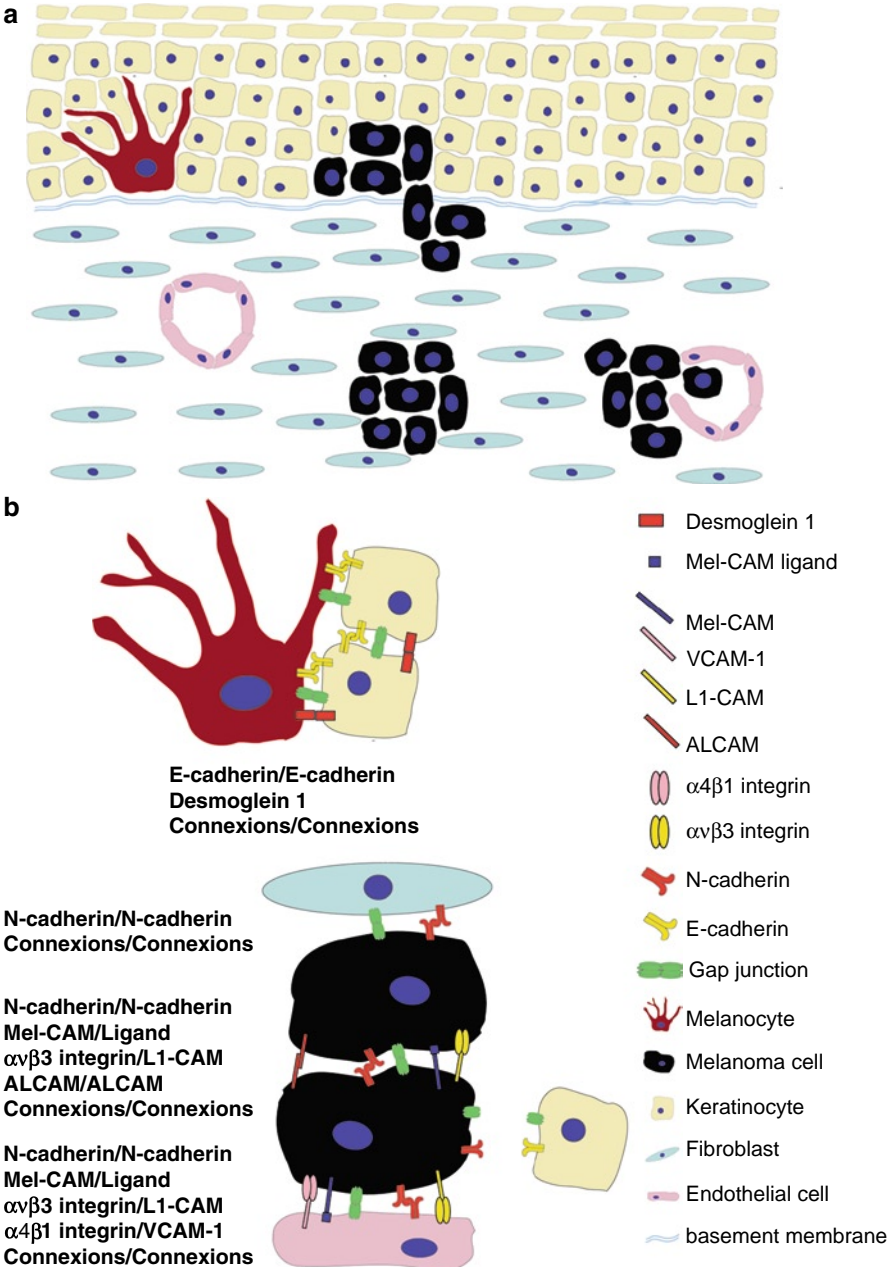


Fig. 9.2 (a) Cell–cell adhesion of melanocytes and melanoma cells. Melanocytes adhere to keratinocytes via E-cadherin and desmoglein, which enables them to communicate with each other through gap junctions with cells in their environment. (b) In melanoma cells E-cadherin is down-regulated. They interact with each other through N-cadherin, Mel-CAM/Mel-CAM ligand, $\alpha v \beta 3$ integrin/L1-CAM, ALCAM/ALCAM, and connexins, with fibroblasts through N-cadherin and connexins, and with endothelial cells through N-cadherin, Mel-CAM/Mel-CAM ligand, $\alpha v \beta 3$ integrin/L1-CAM, $\alpha 4 \beta 1$ integrin/VCAM-1 and connexins

gene is mutated, leading to the expression of a nonfunctional protein (Strathdee 2002). The consequence is abnormal expression and abnormal subcellular localization of cadherin or the components of the cadherin-containing adhesion complex. Further the *CDH-1* gene locus can be epigenetically silenced by hypermethylation, leading to down-regulation of E-cadherin expression which is known from several cancer entities, e.g., hepatocellular carcinoma (Kanai et al. 1997), squamous cell carcinoma (Saito et al. 1998), and thyroid cancer (Graff et al. 1998).

In most cases E-cadherin expression is down-regulated at the transcriptional level. The zinc-finger containing transcriptional repressor Snail, which is a master regulator of neural crest cell specification and melanocyte migration during development in vertebrates, is mainly responsible for the loss of E-cadherin in melanoma (Poser et al. 2001). The level of Snail expression correlates directly with the loss of E-cadherin expression, and forces overexpression of Snail in primary melanocytes down-regulates E-cadherin expression (Poser et al. 2001). Further, the T-box transcription factor Tbx3 is overexpressed in melanoma, which enhances melanoma invasiveness through prevention of E-cadherin expression (Rodriguez et al. 2008). Furthermore, within human melanoma lesions, GLI-2, a mediator of hedgehog signaling, is associated with loss of E-cadherin (Alexaki et al. 2010).

Proteolytic degradation of E-cadherin by matrix metalloproteinases (MMPs) is another mechanism by which E-cadherin mediated cell–cell adhesion can be ablated. In this case, cell surface E-cadherin becomes soluble by cleavage of the extracellular domain, a process known as ectodomain shedding. For melanoma, Adam-10 is responsible for E-cadherin shedding (Billion et al. 2006). (See also Chap. 8).

Recently, a family of microRNAs, such as miR-200a, miR-200b, miR-200c, and miR-205 was reported to control the expression level of E-cadherin during the epithelial-mesenchymal transition. The microRNA targets the transcriptional repressors ZEB1 and ZEB2 of E-cadherin (Gregory et al. 2008; Hurteau et al. 2007). As one example for cancer, loss of miR-200c expression is significantly correlated with early stage T1 bladder tumor progression (Wiklund et al. 2010). Another miRNA, miR-373, induces expression of genes with complementary promoter sequences. It was found that miR-373 induces E-cadherin expression by recognizing a target site in the promoter of the *cdh-1* gene (Place et al. 2008). Whether microRNAs are responsible for regulating cadherins in melanoma is still not known. (See also Chap. 6).

9.1.2

Loss of P-Cadherin During Tumorigenesis

In human skin, P-cadherin is expressed mainly on cells of the epidermal basal layer (Furukawa et al. 1997) and those melanocytes residing in hair follicles (Nishimura et al. 1999). While in melanoma cells loss of full-length P-cadherin was reported (Bachmann et al. 2005), a truncated 50 kDa form of the N-terminal part of P-cadherin was found, which appeared to be secreted from the cells. The secreted P-cadherin is responsible for cell migration and invasion (Bauer et al. 2005, 2006; Bauer and Bosserhoff 2006).

9.1.3

Loss of H-Cadherin During Tumorigenesis

H-cadherin, named for its strong expression in the heart, is an atypical member of the cadherin family, lacking transmembrane and cytosolic domains and possessing a glycosylphosphatidylinositol moiety that anchors H-cadherin into the outer plasma membrane. H-cadherin expression in endothelial cells was demonstrated to be redox sensitive (Joshi et al. 2008). Immunohistochemistry of melanoma tissue samples showed positive H-cadherin staining in the endothelial cells. The melanoma cells themselves showed loss of H-cadherin whereas healthy skin showed staining of melanocytes and keratinocytes of the basal layer of the epidermis (Kuphal et al. 2009).

9.1.4

N-Cadherin Expression During Tumorigenesis

N-cadherin plays a pivotal role in cell adhesion between melanoma cells and both dermal fibroblasts and vascular endothelial cells. During the cadherin class switch loss of E-cadherin expression is accompanied by induced N-cadherin expression, which confers new adhesive properties on the cells (Fig. 9.2). The shift in cadherin profile during melanoma progression has been found not only *in vitro* but also *in vivo* (Hsu et al. 1996; Sanders et al. 1999). Experimentally, melanoma cell migration across fibroblasts is impaired upon addition of an N-cadherin neutralizing antibody (Li et al. 2001). The functional relevance of N-cadherin is to conduct migration and invasion of melanoma cells whereas N-cadherin expression correlates with progression to advanced-stage melanoma. The cell adhesion molecule N-cadherin has been suggested to represent a melanoma progression marker (Watson-Hurst and Becker 2006).

The switch of the cadherin class from E-cadherin to N-cadherin is directly connected. The transcriptional repressor Snail not only regulates E-cadherin repression but also represses the expression of the deubiquitinating enzyme CYLD. Loss of CYLD expression in melanoma in turn led to ubiquitination of Bcl-3 which is a transcriptional regulator of N-cadherin expression (Massoumi et al. 2009).

9.1.5

VE-Cadherin Expression During Tumorigenesis

The term vasculogenic mimicry describes the formation of vascular-like tubular structures and patterned networks through the connection of melanoma cells. The vascular structures are essential for the supply of the tumor. Several key molecules are responsible for the formation and maintenance of the tubular networks and these molecules are also often essential in normal blood vessels. One molecule expressed during vasculogenic mimicry of melanoma cells is VE-cadherin, previously considered to be endothelial cell specific. Analyzing VE-cadherin in detail demonstrated an interaction with EphrinA2 (EphA2), a tyrosine kinase. VE-cadherin engages the membrane-bound ligand of EphA2 and

becomes phosphorylated on its tyrosines at the cytoplasmic domain. The mutual impact of VE-cadherin and EphA2 results in loosening of cell–cell adhesion and allowing for an increase in cell migration, invasion, and vasculogenic mimicry. Further studies describe the role of VE-cadherin for melanoma transendothelial migration. Here, p38 MAP kinase is necessary for increased VE-cadherin mediated junction disassembly important for the migration processes of melanoma cells (Hendrix et al. 2001; Hendrix et al. 2003; Khanna et al. 2010).

9.1.6

Signaling of Cadherins

In contrast to integrins, evidence for cadherin-induced outside-in signaling came into focus only slowly. Over the last 10 years, a number of studies have appeared to agree that signaling cascades emanating from cadherins play an important role in confluency-dependent growth arrest, migration, invasion, and differentiation. Changes in expression or function of cell-adhesion molecules can therefore contribute to tumor progression both by altering the adhesion status and by affecting cell signaling. To date, no enzymatic activity has been attributed to the cytoplasmic tails of adhesion molecules like E-cadherin or N-cadherin. The signaling capability emanates from intracellularly bound kinases and phosphatases that link to the cytoplasmic tail of adhesion receptors (Fig. 9.3).

9.1.6.1

Signaling Cascades of E-Cadherin

Four modes of E-cadherin signaling are known:

1. Modulation of receptor tyrosinase signaling (RTK) (see also Chap. 7)
2. Inhibition of the Wnt signaling pathway (see also Chap. 7)
3. Regulation of cytoplasmic β -catenin signaling
4. Regulation of signaling through Rho GTPases

One way by which E-cadherin transmits growth-inhibiting outside-in signals appears to follow a strikingly similar scheme to that of the integrins. By using an immortalized non-tumorigenic keratinocyte cell line, HaCaT, as a model system, Pece and Gutkind (2000) provide evidence that the assembly of calcium-dependent adherens junctions leads to a rapid and remarkable increase in the state of activation of MAPK and that this event is mediated by E-cadherin. Furthermore, it was found in these studies about HaCaTs that E-cadherin stimulates the MAPK pathway through ligand-independent activation of receptor tyrosine kinases, in particular EGF-receptors (Pece and Gutkind 2000). They speculated that upon adherens junction formation, signals emanating as a result of the E-cadherin-EGFR interaction, may be involved in maintaining the functional and structural integrity of quiescent epithelia and, as a function of the adhesion status of the cells, possibly in promoting epithelial cell differentiation rather than proliferation. In contrast, another group detected signaling cascade inhibition through EGF-receptor/E-cadherin complex formation in melanoma and breast cancer cells (Qian et al. 2004). Unfortunately, most of the literature on E-cadherin

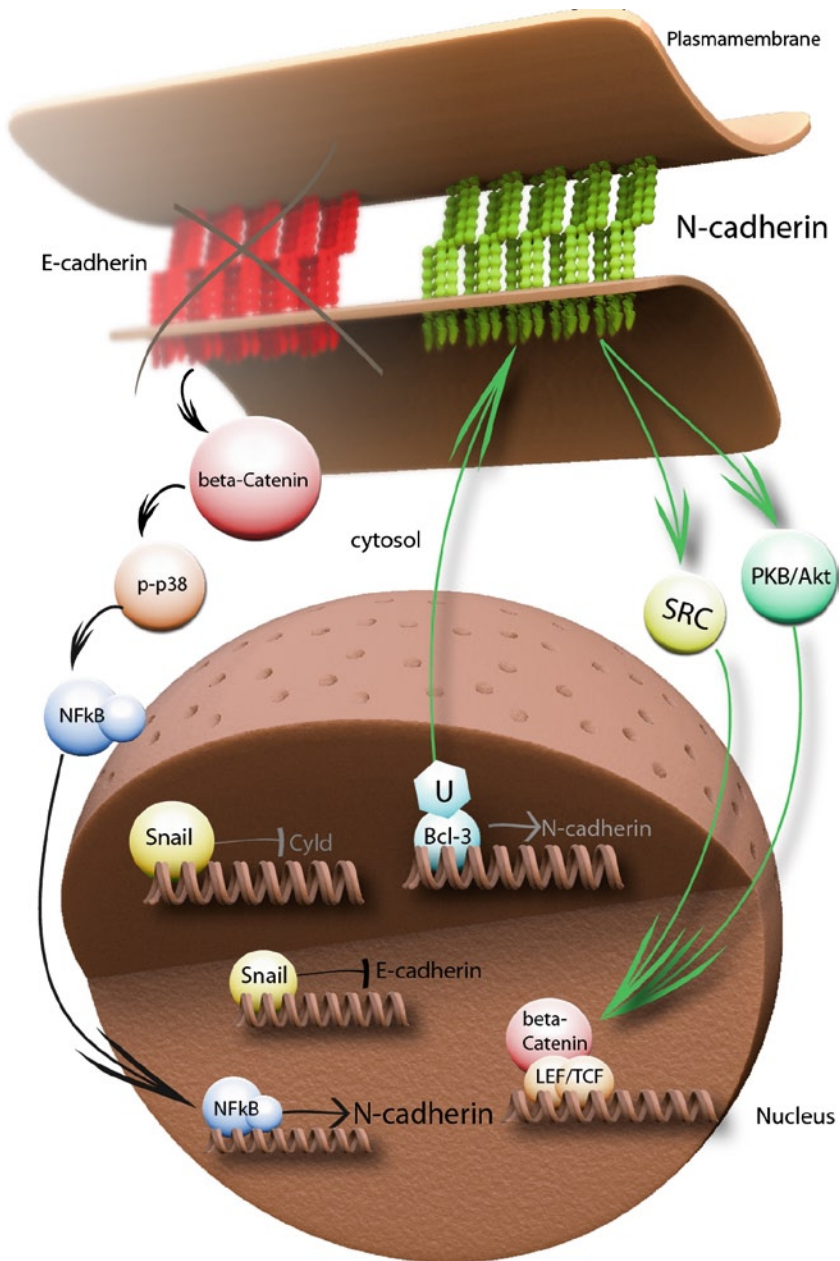


Fig. 9.3 Schematic depiction of cadherin signaling in melanoma. The transcriptional repressor Snail inactivates E-cadherin expression in melanoma. With the loss of E-cadherin cytosolic beta-catenin activates the MAPKinase p38 which stimulates the transcriptional activity of NFKappaB. NFKappaB has N-cadherin as target gene. Additionally, Snail represses the expression of the tumor suppressor Cyld which in turn leads to ubiquitination of Bcl-3 which also has N-cadherin as target gene. The overexpression of N-cadherin activates signaling cascades of SRC and PKB/Akt which leads to tumor progression (Illustration R.J. Bauer)

signaling does not cover melanoma. Studies on keratinocytes and other cancer cell types revealed that the E-cadherin complex associates and cooperates with an EGF-Receptor family member to activate the PI3K/Akt pathway in a Src-family kinase-dependent manner (Muller et al. 2008; Perrais et al. 2007). (See also Chap. 7)

Some studies showed that homophilic ligation of E-cadherin signals directly through Rho GTPase activity (Braga 2000; Braga et al. 1997). Loss of E-cadherin in melanoma may involve changes in the organization of the cytoskeleton which is exerted by members of the Rho family. They control not only the cytoskeletal organization but also at the same time cell motility, migration, and tumor progression to malignancy. E-cadherin suppresses RhoA activity in melanoma by activating p190RhoGAP (Molina-Ortiz et al. 2009). E-cadherin overexpression led to association of p190RhoGAP and p120^{ctn} on the plasma membrane where E-cadherin bounds p120^{ctn}.

In addition to its role in adhesion, nuclear β -catenin is involved in Wnt signal transduction, and it interacts with transcription factors of the leukocyte enhancer factor (LEF)/T-cell factor (TCF) family to regulate transcription of target genes implicated in cell growth control, such as cyclinD1 and c-myc (van Noort and Clevers 2002). By sequestering β -catenin at the cell surface, E-cadherin has been shown to antagonize nuclear β -catenin signaling pathways and to induce growth inhibition (Gottardi et al. 2001; Shtutman et al. 1999). Furthermore, β -catenin bound to E-cadherin inhibits phosphorylation of p38 and prevents activation of NF κ B. Unbound cytoplasmic β -catenin activates the signaling pathway ending at transcriptional activation of N-cadherin expression in melanoma cells (Kuphal et al. 2004). In general it was shown by Onder et al. (2008) that loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. The publication presents ~84 of 617 genes differentially expressed in shE-cadherin human breast epithelial cells (HMLE). They presented, e.g., *twist* and *TCF-8* among other 19 transcription factors as up-regulated after loss of E-cadherin.

9.1.6.2

Signaling Cascades of N-Cadherin

N-cadherin mediated intercellular interactions promote survival and migration of melanoma cells through activation of cytoplasmic signaling cascades. The Src family kinases are involved in the regulation of N-cadherin-mediated cell adhesion and signaling during, e.g., melanoma cell transendothelial migration. Src is localized at the heterotypic contacts of N-cadherin and becomes activated when melanoma cells are transmigrating across the endothelium. Activated Src has the Tyrosine-860 at the cytoplasmic domain of N-cadherin as target site for phosphorylation. The phosphorylation leads to disruption of β -catenin binding followed by nuclear translocation of this molecule to activate gene transcription of genes responsible for proliferation (Qi et al. 2006). N-cadherin mediates cell adhesion activated antiapoptotic protein Akt/PKB and subsequently increases β -catenin and inactivates proapoptotic factor Bad (Li et al. 2001).

9.1.7

Desmosomes/Hemidesmosomes

Desmosomes, composed of desmogleins and desmocollins, are localized spot-like adhesions randomly arranged on the lateral sides of plasma membranes and are also members

of the cadherin family. The extracellular domain of the desmosome is called the Extracellular Core Domain (ECD) or the Desmoglea, and is bisected by an electron-dense midline where the desmoglein and desmocollin proteins bind to each other. On the cytoplasmic side of the plasma membrane, there are two dense structures called the Outer Dense Plaque (ODP) and the Inner Dense Plaque (IDP). In the ODP the cytoplasmic domains of the cadherins desmoglein and desmocollin attach to desmoplakin via plakoglobin and plakophilin, while in the IDP desmoplakin attaches to the intermediate filaments, like keratine filaments.

A number of melanoma cell lines synthesize, in the absence of desmosomes, the desmosomal cadherin desmoglein 2 (Dsg2) as a frequent plasma membrane glycoprotein that is not assembled into any junction but is dispersed over large parts of the cell surface. Indeed, in tissue microarrays, Dsg2 has been demonstrated in a sizable subset of nevi and primary melanomas (Rickelt et al. 2008). In contrast, Dsg1, Dsg3, and desmocollins 1–3, were absent in the analyzed melanoma cell lines but plakoglobin and plakophilin3 were also expressed in several melanoma cell lines (Schmitt et al. 2007). Future studies will have to clarify the diagnostic and prognostic significance of these different adhesion protein subtypes.

9.2 Integrins

Integrins are transmembrane adhesion receptors localized at cell–matrix contact sites where they link ECM (extracellular matrix) components, e.g., vitronectin, fibronectin, laminin, osteopontin, or collagen, to the actin cytoskeleton and interact with multiple structural and signaling molecules including talin, kindlin, paxillin, vinculin, α -actinin, FAK (focal adhesion kinase), ILK (integrin linked kinase), Rho GTPases, and SHC (Berrier and Yamada 2007; Papisheva and Heisenberg 2010). The latter are important mediators downstream of integrins by which they interact either directly or indirectly to effect adhesion-dependent responses (Playford and Schaller 2004). The metastatic transformation of melanocytes is associated with altered expression of integrins, which transduce signals upon ligation to ECM proteins that regulate tumor growth and metastasis, apoptosis, differentiation as well as tumor angiogenesis. Integrin receptors are functional dimers of α - and β -integrin subunits, which each have a large ectodomain, a single transmembrane domain, and a generally short cytoplasmic tail (exceptional $\beta 4$ integrin). The combination of different α - and β -subunits determines the substrate specificity of the dimer (Danen and Sonnenberg 2003). There are at least 18 known α -chains and 8 β -chains, allowing for at least 24 unique heterodimers.

The pattern of integrins on the cell surface is usually very specific which makes the cell fit perfectly into its surrounding environment. Importantly, integrin expression patterns differ considerably *in vitro* versus *in vivo*. Thus, *in vitro* studies may not translate into the *in vivo* situation.

Several publications have shown that the expression levels mainly of $\alpha v \beta 3$, $\alpha 2 \beta 1$, $\alpha 3 \beta 1$, $\alpha 4 \beta 1$, and $\alpha 5 \beta 1$ appears to increase from primary melanomas to metastatic melanoma tissue sections, whereas there was a significant decrease in $\alpha 1 \beta 1$, $\alpha 2 \beta 1$, and $\alpha 6 \beta 1$ expression levels in metastatic melanoma compared to primary melanoma (Friedl et al. 1998; Natali et al. 1993; Schadendorf et al. 1993). Although many integrins have been

implicated in mediating melanoma growth and metastasis, perhaps none have been studied as much as the vitronectin receptor, $\alpha v\beta 3$ (Danen et al. 1995; Johnson 1999; Mortarini and Anichini 1993; Seftor et al. 1999). $\alpha v\beta 3$ integrin adheres to vitronectin, fibronectin, laminin, collagen, and osteopontin. Binding fibronectin and vitronectin induces the expression of MMP-2, which is able to degrade the collagen of the basement membrane (Felding-Habermann et al. 2002). Furthermore, osteopontin's RGD-Sequence (Arg-Gly-Asp) has high binding affinity and specificity to $\alpha v\beta 3$. As aggressiveness of melanoma has been associated with high osteopontin expression (Sieg et al. 2000), this interaction of $\alpha v\beta 3$ and osteopontin is important for melanoma progression. Interaction between $\alpha v\beta 3$ and extracellular matrix molecules serves to promote cell attachment, spreading and migration. $\alpha v\beta 3$ integrin also undergoes heterophilic binding with two members of the immunoglobulin superfamily of cell adhesion molecules, PECAM-1 and L1. The αv subunit is widely expressed on melanomas regardless of disease stage. This stands in contrast to the $\beta 3$ subunit, which is predominantly expressed on melanoma cells in the vertical growth phase. The onset of $\beta 3$ integrin expression is one of the most specific markers of the transition from radial growth phase to vertical growth phase of melanoma (Albelda et al. 1990; Danen et al. 1995; Natali et al. 1997). Although many studies on human melanoma cell lines have correlated $\alpha v\beta 3$ integrin expression with progression and metastasis, *in vivo* studies are less clear.

9.2.1

Integrin Signaling in Melanoma

Apart from being involved in the attachment of cells to the ECM, integrins are also responsible for signaling between the cells and the environment. Signaling works bidirectionally: "Outside-in signaling" can control behavior, proliferation, cell polarity, cell growth, and migration. "Inside-out signaling" on the other hand changes the integrins from a passive, weak binding state into an active, adhesive state and alters the interaction of the receptors with the extracellular environment. Integrins are receptors for cell movement in response to binding to ECM of the basement membrane or connective tissue or plasma membrane receptors expressed on endothelial cell surfaces. Additionally, integrins bind cytoplasmic adaptor proteins of the actin-myosin filaments and create a plasticity that allows the cell to move. In summary, integrins are bivalent linker proteins, binding simultaneously to extracellular ligands as well as cytoplasmic proteins including intracellular signaling molecules. They influence, for example, tyrosine kinases, serine/threonine kinases, phosphoinositides, and signaling cascades which determine the fate of a cell, letting it grow, proliferate, or die whenever it is necessary in the context of the whole organism. This paragraph introduces some of the most important and best studied proteins which are known to interact with integrins in melanoma.

There is the non-receptor protein tyrosinase kinase FAK (Focal adhesion kinase) (Fig. 9.4) that co-localizes with integrins in focal adhesions. FAK becomes phosphorylated and then controls processes like cell spreading, proliferation, motility, vasculogenic mimicry, and survival (Schaller 2001). Proteins like c-SRC, SHC, CSK, PI3K, and GRB2 are known to interact with FAK to transfer the signaling into the cytoplasm and to link

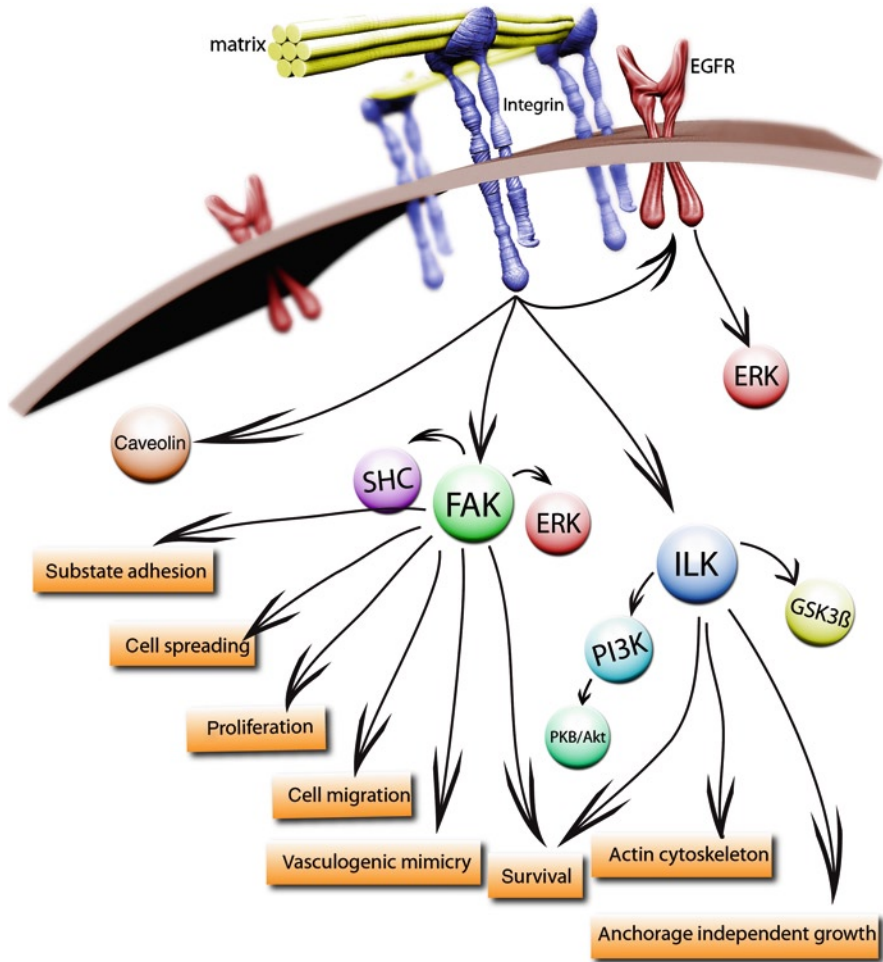


Fig. 9.4 Schematic depiction of the signaling pathways leading from integrins to focal adhesion kinase (*FAK*) and integrin linked kinase (*ILK*), respectively, and further reactions of the cell (Illustration R.J. Bauer)

FAK signaling also to MAPKinses (Chakraborty et al. 2002). (See also Chap. 7). FAK expression seems to be required in melanoma cells for substrate adhesion. It has been shown that in melanoma FAK is constitutively active and that it is essential for maintaining adhesiveness in melanoma cells (Hamamura et al. 2008; Kahana et al. 2002).

Furthermore, the integrin-linked kinase (ILK), a serine/threonine kinase, is implicated in connecting cell-extracellular matrix interaction and growth factor signaling to cell survival, cell migration, invasion, anchorage-independent growth, angiogenesis, and epithelial-mesenchymal transition. It has been shown that strong ILK expression was significantly associated with melanoma thickness, migration, and invasion (Wong et al. 2007). Increased

expression of integrin-linked kinase is correlated with melanoma progression and poor patient survival (Dai et al. 2003). ILK directly phosphorylates PKB/Akt and glycogen synthase kinase-3 (GSK-3 β), which is inactivated upon phosphorylation (Delcommenne et al. 1998; Troussard et al. 1999). SHC is another protein which is implicated in integrin signaling. It is an adaptor protein capable of binding phosphotyrosine-containing sequences. So far, studies have demonstrated that SHC signaling is involved in pathways, which play a role in the development of malignancies like c-Myc activation (Gotoh et al. 1997), survival signaling (Friedmann et al. 1996; Sakai et al. 2000), cytoskeletal organization, and mitogenic signaling through RAS. It has been proposed that SHC is a substrate for FAK.

Also the ERK/MAPKinase cascade is a pathway in which integrin-mediated adhesion is involved. In the ERK pathway, various stimuli of many important integrin signaling molecules like FAK or SHC converge and are able to influence nearly every profound cellular activity (Meier et al. 2005).

Epidermal growth factor receptor (EGFR) is also activated by integrins to generate cellular responses like adhesion dependent cell survival and proliferation in response to ECM. Subsequently, integrin-mediated EGFR activation induces ERK/MAPKinase signaling (Howe et al. 2002; Jost et al. 2001). Furthermore, Caveolin-1 (CAV1) is the main structural component of caveolae, which are plasma membrane invaginations that participate in vesicular trafficking and signal transduction events. Following integrin activation, B16F10 cells expressing CAV1 display reduced expression levels and activity of FAK and Src proteins. Furthermore, CAV1 expression markedly reduces the expression of integrin β 3 in B16F10 melanoma cells. These findings provide experimental evidence that CAV1 may function as an antimetastatic gene in malignant melanoma (Trimmer et al. 2010).

9.3

Immunoglobulin Gene Superfamily of Cell Adhesion Molecules (CAMs)

Whereas normal melanocytes express few cell–cell adhesion receptors of the immunoglobulin gene superfamily of cell adhesion molecules (CAMs), melanoma cells show an increase in expression of Melanoma Cell Adhesion Molecule (MCAM, Mel-CAM, MUC18, CD146), L1 Cell Adhesion Molecule (L1-CAM, CD171), Activated Leukocyte Cell Adhesion Molecule (ALCAM, CD166), Vascular Cell Adhesion Molecule 1 (VCAM-1, CD106), Intercellular Cell Adhesion Molecule 1 (ICAM-1, CD54), and Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 (CEACAM-1, CD66a) (reviewed in Haass et al. 2005).

9.3.1

Melanoma Cell Adhesion Molecule (MCAM, Mel-CAM, MUC18, CD146)

Mel-CAM mediates homologous and heterologous interactions between melanoma cells and endothelial cells respectively via a heterophilic Ca^{2+} -independent adhesion to

a currently unidentified ligand (Johnson et al. 1997; Shih et al. 1997a, b). In melanocytic cells expression of Mel-CAM is first found in nevi, when the cells have separated from the epidermal keratinocytes and have migrated into the dermis (Kraus et al. 1997; Shih et al. 1994). With progression to malignancy, Mel-CAM expression gradually increases and is highest in metastatic melanoma cells (Johnson et al. 1996; Lehmann et al. 1987, 1989; Shih et al. 1994; Xie et al. 1997). The important role of Mel-CAM in melanoma progression was demonstrated in several experimental studies (reviewed in Haass et al. 2005).

A recent study evaluated tissue arrays of primary and metastatic melanomas (Pearl et al. 2008). In patients meeting the current criteria for sentinel lymph node dissection, both Mel-CAM expression positivity and intensity were independently predictive of survival and development of lymph node disease in primary melanoma over and above established markers of prognosis, such as Breslow thickness. Mel-CAM-negative patients had a 5-year survival of 92% compared with 40% for Mel-CAM positive (Pearl et al. 2008).

9.3.2

L1-Cell Adhesion Molecule (L1-CAM, CD171)

L1-CAM, originally described as a neuronal cell adhesion molecule, has also been detected in a number of other non-neuroendocrine tissues and in several malignant tumors, including melanoma (Nolte et al. 1999; Thies et al. 2002b). L1-CAM mediates adhesion both *via* homophilic (L1-CAM-L1-CAM) and heterophilic (L1-CAM- $\alpha_v\beta_3$ integrin) mechanisms (Hortsch 1996). In melanoma/melanoma cell and in melanoma/endothelial cell interactions L1-CAM binds to $\alpha_v\beta_3$ integrin (Montgomery et al. 1996). The interaction of L1-CAM and $\alpha_v\beta_3$ integrin plays an important role in transendothelial migration of melanoma cells (Voura et al. 2001) whereas overexpression of L1-CAM promotes conversion from radial to vertical growth phase melanoma without up-regulation of $\alpha_v\beta_3$ integrin expression (Meier et al. 2006). There is an increase in L1-CAM immunoreactivity in melanomas and metastases of melanoma compared to acquired melanocytic nevi (Fogel et al. 2003). A study that systematically identified novel melanoma-specific genes confirmed that L1-CAM is not expressed in normal skin and melanocytic nevi, but is highly and differentially expressed in primary melanoma tissues and melanoma lymph node metastases (Talantov et al. 2005). A recent study, evaluating the specimens of nevi, primary melanomas, sentinel lymph nodes, and distant metastases, showed that L1-CAM can serve as a highly sensitive and specific diagnostic marker for melanoma (Thies et al. 2007). A 10-year retrospective biomarker study, evaluating 100 melanoma specimens, showed that the expression of L1-CAM in human primary cutaneous melanoma is significantly associated with metastatic spread and that L1-CAM expression is an independent predictor for the risk of metastasis (Thies et al. 2002b).

9.3.3

Activated Leukocyte Cell Adhesion Molecule (ALCAM, CD166)

ALCAM is involved in homophilic (ALCAM-ALCAM) (Degen et al. 1998) and heterophilic (ALCAM-CD6) (Patel et al. 1995) cell–cell adhesion interactions. ALCAM is expressed in metastatic human melanoma cells, whereas it is absent in non-metastatic cells (Degen et al. 1998). Immunohistochemistry on a series of common nevi, primary melanomas, and melanoma metastases revealed that ALCAM expression correlates with melanoma progression (van Kempen et al. 2000). ALCAM is therefore proposed to be a molecular melanoma progression marker. It has been shown that the intact cell adhesion function of ALCAM favored primary tumor growth and represented a rate-limiting step for tissue invasion, which supported the view that dynamic control of ALCAM plays an important role in progression (van Kempen et al. 2004). An immunohistochemical biomarker study, evaluating tissue microarrays showed that a significantly greater percentage of melanomas (combined primary and metastatic) than nevi contained cells that expressed ALCAM (Klein et al. 2007).

9.3.4

Intercellular Adhesion Molecule-1 (ICAM-1, CD54)

ICAM-1 can be induced in a cell-specific manner by several cytokines, e.g., TNF- α (tumor necrosis factor-alpha), IL-1 (interleukin-1), and IFN- γ (interferon-gamma). The ligands of ICAM-1 are $\alpha_L\beta_2$ (lymphocyte function-associated antigen 1, LFA-1) and Mac1 on lymphocytes (van de Stolpe and van der Saag 1996). ICAM-1 expression correlates with melanoma progression and increased risk of metastasis (Johnson et al. 1989). Its expression in melanoma is stronger than in common nevi and increases with the Breslow index in primary melanomas (Natali et al. 1990, 1997; Schadendorf et al. 1993, 1995). The observation that stage I patients with ICAM-1 positive melanomas had a significantly shorter disease-free interval and overall survival than those with ICAM-1 negative tumors (Natali et al. 1997) and that the suppression of ICAM-1 in an animal model reduced the metastatic capacity (Miele et al. 1994), supported the role of ICAM-1 in melanoma progression and metastasis. However, the specific role of ICAM-1 in melanoma progression remains to be determined. Expression of ICAM-1 may promote aggregate formation with leucocytes, which can enhance survival in the vascular system and encourage extravasation (Aeed et al. 1988). On the other hand ICAM-1 is shed from melanoma cells (Giavazzi et al. 1992) – possibly in a form that inhibits lymphocyte-tumor cell interaction and thus contribute to tumor survival (Becker et al. 1993).

9.3.5

Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (CEACAM-1, CD66a)

CEACAM1 is involved in intercellular adhesion and subsequent signal transduction events in a number of epithelia. In epithelial cells CEACAM1 is believed to act as a

growth suppressor, since its expression was shown to be lost or significantly down- or dys-regulated in carcinomas of liver, prostate, endometrium, breast, and colon (reviewed in Haass et al. 2005). On the other hand CEACAM1 is up-regulated in non-small cell lung cancer (Sienel et al. 2003). CEACAM1 interacts with the β_3 integrin subunit via the CEACAM1 cytoplasmic domain. CEACAM1 and the β_3 integrin subunit co-localize at the tumor-stroma interface of invading melanoma masses, suggesting that CEACAM1-integrin β_3 interaction plays a role in melanoma cell migration and invasion (Brummer et al. 2001). The expression of CEACAM1 in primary melanomas is associated with the subsequent development of metastatic disease (Thies et al. 2002a). Furthermore, the overexpression of CEACAM1 in CEACAM1-negative melanocytic cells and melanoma cell lines increases the migratory and invasive growth potentials *in vitro* (Ebrahimnejad et al. 2004) supporting the role of CEACAM1 in melanoma progression and metastasis. A recent study, evaluating the specimens of nevi, primary melanomas, sentinel lymph nodes, and distant metastases, showed that CEACAM1 can serve as a highly sensitive and specific diagnostic marker for melanoma (Thies et al. 2007).

9.4

Gap Junctions/Connexins

Connexins belong to a family of transmembrane proteins that form gap junctions (GJs), cell–cell junctions that are essential for intercellular communication. Gap junctional intercellular communication (GJIC) in the skin is important for proliferation, differentiation, and homeostasis as well as barrier function and the recruitment of inflammatory cells, and is thus a critical factor in the life and death balance of cells (Djalilian et al. 2006; Langlois et al. 2007; Maass et al. 2004; Man et al. 2007) (reviewed in Kretz et al. 2004; Mese et al. 2007). Furthermore, GJIC is critical in keratinocyte-melanocyte interaction (Hsu et al. 2000). GJs form channels between adjacent cells allowing the intercellular transport of small metabolites, second messengers, and ions (Loewenstein 1981; Spray 1994). Each GJ channel consists of two hemi-channels called connexons, each formed by six connexins (reviewed in Richard 2000). Twenty-one connexins have been identified, ten of those are expressed in the skin (Di et al. 2001; Willecke et al. 2002). GJs can be homotypic, heterotypic, homomeric, and heteromeric (reviewed in Richard 2000). A connexon is homomeric if it is composed of six identical connexin subunits (e.g., Cx32 only), or heteromeric if it is composed of more than one connexin species (e.g., Cx32 and Cx43 and/or others). Channels are homotypic, if both connexons are homomeric of the same type, heterotypic if homomeric connexons are of different types, and heteromeric if both connexons are heteromeric. Not all connexins are equally compatible at forming a connexon – even though they may co-exist in the same cell (reviewed in Haass et al. 2004). The type of connexin forming GJ channels influences their selectivity and thereby controls the specificity of GJIC. For example, channels formed by Cx26 prefer cations, while those formed by Cx32 prefer anions (Brisette et al. 1994; Elfgang et al. 1995; Veenstra 1996). Thus, the up- or down-regulation of a certain connexin in a tissue may change its GJIC considerably. In addition, connexins can also form hemichannels which have been shown to be able

to exchange molecules with the extracellular microenvironment. These hemichannels are relevant for signal propagation and especially for calcium homeostasis (reviewed in Evans et al. 2006).

9.4.1

Connexins Play Multiple Roles in Cancer

Loss of gap junctional activity and/or down-regulation of connexins has been reported both in cell lines as well as in tissues of many tumor types, such as hepatocellular carcinoma, gastric carcinoma, prostate cancer, lung cancer, glioma, mammary carcinoma, basal cell carcinoma, squamous cell carcinoma and, last but not least, melanoma. This phenomenon was first observed almost half a century ago (Loewenstein and Kanno 1966) and nicely summarized in a number of recent review articles (Cronier et al. 2009; Mesnil et al. 2005; Naus and Laird 2010). The type of connexins lost during tumor progression varies according to tumor type. In the 1980s and 1990s a series of studies were published showing that reagents and/or oncogenes that promote tumor onset or progression frequently inhibit GJIC or down-regulate connexin expression (Atkinson et al. 1981; Lampe 1994; Trosko et al. 1990). The role of connexins as potential tumor suppressors was also shown in gene knockdown studies (Shao et al. 2005). Correspondingly, ectopic expression of connexins in tumors restored functional communication and reduced tumor proliferation and growth both *in vitro* and *in vivo* (reviewed in Naus and Laird 2010). Importantly, ectopic expression of connexins partially differentiated transformed cells (Hellmann et al. 1999; Hirschi et al. 1996; McLachlan et al. 2006; Zhu et al. 1991). Moreover, functional abrogation of connexins, using antisense or dominant negative mutant approaches, have demonstrated an enhancement of the malignant phenotype in several tumor types; such as Cx26 in HeLa-cells (Dufrot-Dancer et al. 1997); Cx32 in hepatocellular carcinoma (Dagli et al. 2004); Cx43 in lung cancer (Avanzo et al. 2004); Cx43 in glioma (Omori and Yamasaki 1998) and Cx43 in bladder carcinoma (Krutovskikh et al. 1998). Finally, Cx32 knock-out mice have an increased incidence of tumor onset when challenged with carcinogens (King and Lampe 2004a, b; Moennikes et al. 2000; Temme et al. 1997).

However, despite all this evidence of connexins being tumor suppressors, this appears to be mainly true for the earlier steps of cancerogenesis. The following model is nicely summarized in a recent review (Cronier et al. 2009): For the step from primary to invasive tumors there is need for disruption of intercellular junctions including GJs, consistent with the model that connexins are tumor suppressors. However, for the tumor cell dissemination and metastasis steps, increased cell contacts and communication are needed in order to be able to interact with the tumor stroma – especially between cancer cells and endothelial cells. Therefore, connexins might be better classified as conditional tumor suppressors that modulate cell proliferation, as well as adhesion and migration (Naus and Laird 2010).

9.4.1.1

Cx43 in Cancer

Cx43 is decreased in prostate cancer (Tsai et al. 1996), mammary cancer (Hirschi et al. 1996), glioma (Huang et al. 1999), lung cancer (Jinn et al. 1998; Zhang et al. 1998), bladder

carcinoma (Krutovskikh et al. 2000), cervical carcinoma (King et al. 2000) and various skin cancers including melanoma (Haass et al. 2006; Tada and Hashimoto 1997; Wilgenbus et al. 1992). Electron microscopy investigations have shown that basal and squamous cell carcinomas do not have fully developed GJs, and that Cx43 is not restricted to these poorly developed GJs but is present in the cytoplasm (Tada and Hashimoto 1997). In several cancers, Cx43 acts as a tumor suppressor gene with loss of Cx43 contributing to metastasis (Czyz 2008; Gershon et al. 2008; Shen et al. 2007). Functional abrogation of Cx43 enhances the malignant phenotype in lung cancer (Avanzo et al. 2004), glioma (Omori and Yamasaki 1998) and bladder carcinoma (Krutovskikh et al. 1998).

In contrast to other cancers, hepatocellular carcinoma is associated with an induction of Cx43, which is, however, localized in the cytoplasm, and thus is not involved in GJIC (Krutovskikh et al. 1994). The loss of GJIC might help the tumor cells to survive, as GJIC has been shown to spread cell-killing signals, most likely Ca^{2+} ions (Krutovskikh et al. 2002).

Conversely, expression of Cx43 has also been shown to increase tumor metastasis in breast cancer, glioma as well as in melanoma through increased attachment and communication with the vascular endothelium (Bates et al. 2007; Cotrina et al. 2008; el-Sabban and Pauli 1991, 1994; Elzarrad et al. 2008; Kanczuga-Koda et al. 2006; Lin et al. 2002; Pollmann et al. 2005).

9.4.1.2

Cx32 in Cancer

Cx32 is down-regulated in gastric carcinoma (Uchida et al. 1995), lung cancer (Jinn et al. 1998) and hepatocellular carcinoma (Eghbali et al. 1991; Krutovskikh et al. 1994; Loewenstein and Rose 1992; Yamaoka et al. 1995). In the latter case, the remaining Cx32 is localized in the cytoplasm or in the plasma membrane free from contact with other cells. In addition, it was found that there was no mutation in the coding sequence of Cx32 in hepatocellular carcinoma; instead it appears that the aberrant localization of Cx32 is a consequence of the disruption of Cx32 gap junction plaque formation (Krutovskikh et al. 1994). Functional abrogation of Cx32 enhances the malignant phenotype in hepatocellular carcinoma (Dagli et al. 2004). Cx32 knock-out mice have an increased incidence of tumor onset when challenged with carcinogens (King and Lampe 2004a, b; Moennikes et al. 2000; Temme et al. 1997). In contrast to most other tumors, Cx32 is up-regulated in some breast cancer cells (Saunders et al. 2001).

9.4.1.3

Cx26 in Cancer

Whereas in mammary carcinoma cells, there is a down-regulation of both Cx43 and Cx26 (Hirschi et al. 1996), in human basal cell carcinoma Cx43 is down-regulated but there is an induction of Cx26 (Haass et al. 2006; Wilgenbus et al. 1992). Cx26 is also highly expressed in HeLa-cells, where its functional abrogation enhances the malignant phenotype (Dufлот-Dancer et al. 1997).

9.4.1.4

Connexins in Melanoma

To date, there is little known about GJIC in melanoma. Preliminary evidence indicates that Cx43 may be a tumor suppressor in malignant melanoma (Su et al. 2000). Melanocytes, but not melanoma cells, are able to communicate with keratinocytes via GJIC. Instead, melanoma cells communicate amongst themselves and with fibroblasts (Hsu et al. 2000), and it appears that the switch in communication partners coincides with the cadherin switch (see above). Forced expression of E-cadherin by adenoviral gene transfer into N-cadherin-expressing melanoma cells restored gap junctional compatibility with keratinocytes (Hsu et al. 2000), suggesting that the gain of N-cadherin with the concurrent loss of E-cadherin (see above) facilitates GJ formation with fibroblasts and endothelial cells (Hsu et al. 2000). Additionally, GJ formation in melanoma cell lines appears to require MCAM (Satyamoorthy et al. 2001). The existence of GJIC between melanoma cells may contribute to the bystander effect of gene therapies, in which some melanoma cells in a population were transfected with the herpes simplex virus thymidine kinase gene, but nevertheless also adjacent non-transfected melanoma cells were killed by treatment with the antiviral drug, ganciclovir (Burrows et al. 2002).

In the early steps of melanoma progression there is a decrease in Cx43 in human melanoma cells (Hsu et al. 2000). However, melanoma cells expressing higher levels of Cx43 show increased coupling to vascular endothelial cells (el-Sabban and Pauli 1991) and the ability of tumor cells to metastasize appears to correlate with the ability of tumor cells to communicate with endothelial cells (Pollmann et al. 2005). A recent study shows high levels of Cx43 protein expression in metastatic melanoma cell lines and that loss of PAR-1 expression results in the loss of Cx43 (Villares et al. 2009). This finding does not support the role of Cx43 acting as a tumor suppressor gene in melanoma, but rather suggests another possible mechanism by which PAR-1 contributes to invasion and metastasis in melanoma by regulating Cx43.

Cx26 is up-regulated in the highly aggressive BL6 sub-line of B16 mouse melanoma cells, but not in the less aggressive F10 sub-line (Ito et al. 2000). F10 cells transfected with wild-type Cx26 exhibited similar metastatic behavior to the BL6 cells. Correspondingly, BL6 cells transfected with a dominant-negative Cx26 mutant showed the less aggressive behavior characteristic of F10 cells. Moreover, no Cx26 expression was found in human melanomas within the epidermis, but an up-regulation in the areas invading into the dermis (Ito et al. 2000). In contrast, we have shown in immunofluorescence studies, that all areas of melanomas lack Cx26, Cx30, and Cx43 expression (Haass et al. 2006, 2010) – similar to our findings in Merkel cell carcinoma (Haass et al. 2003a). This discrepancy may be due to previously described cross-reactivities of the Cx26 antibody used by Ito and colleagues.

9.4.1.5

Connexins in the Epidermal Tumor Environment of Melanoma

Melanoma depends on, interacts with, and reacts to its stroma, including fibroblasts, extracellular matrix, endothelial cells, and immune cells (reviewed in Villanueva and Herlyn

2008). (See also Chap. 14). In addition to the interaction with the tumor stroma – melanoma progression exhibits a significant impact on the epidermal tumor microenvironment: the multilayered epithelium of the skin (Haass et al. 2010). While in the vast majority of the investigated melanoma samples Cx26 and Cx30 were up-regulated in the epidermal tumor microenvironment, in the epidermis adjacent to melanocytic nevi these connexins were not up-regulated in most cases. This matched the results of a previous study, in which we compared the expression patterns of Cx26 and Cx30 in benign versus malignant skin tumors, including Merkel cell carcinoma, squamous cell carcinoma, and basal cell carcinoma, demonstrating that only malignant tumors influence the epidermal tumor microenvironment, while benign tumors did not (Haass et al. 2003a, 2006).

The results of our recent study, which included dysplastic nevi as well as thin melanomas which are often difficult to distinguish (reviewed in Haass and Smalley 2009), suggest that membrane expression of Cx26 and Cx30 in the epidermal tumor microenvironment may be a useful diagnostic aid for the distinction of melanomas and melanocytic nevi (Haass et al. 2010). As neither Cx26 nor Cx30 are expressed in the melanoma itself, but both are induced in its tumor microenvironment, they may be useful complementary melanoma markers.

Moreover, we showed a significant positive correlation between melanoma thickness and Cx26 and Cx30 up-regulation in the immediate epithelial tumor microenvironment (Haass et al. 2010). As we did not observe this correlation with benign tumors which can also gain substantial size (Haass et al. 2006), or melanocytic nevi (Haass et al. 2010), this does not simply reflect epidermal damage due to physical stress through the tumor. Therefore it is more likely that melanomas secrete soluble substances resulting in an alteration of the epidermis causing up-regulation of Cx26 and Cx30.

Cx26 and Cx30 up-regulation in the epidermal tumor microenvironment did not correlate with the proliferative index of the melanoma cells, but correlated significantly with the proliferative index in the epidermis. It is unclear whether this is a cause or consequence of the increased expression of connexins 26 and 30 in the epidermis. An answer to this question might be provided by a study in which transgenic mice expressing Cx26 ectopically by an involucrin promoter show increased levels of proliferative cells in the epidermis (Djalilian et al. 2006), suggesting that Cx26 influences keratinocyte proliferation and not *vice versa*. Interestingly, Cx26 overexpressing mice showed a delay in wound healing, which needs to be explored with regards to ulceration, a biomarker associated with very poor prognosis for melanoma patients (Balch et al. 2001). In our study, all melanomas with ulceration showed Cx26 (and Cx30) expression in all layers of the epidermal tumor microenvironment (Haass et al. 2010). McCarty and colleagues hypothesized that induction of angiogenesis by the hyperplastic epithelium could stimulate growth and progression of melanoma (McCarty et al. 2003). This suggests a positive feedback mechanism: tumor cells induce alterations in keratinocytes, which results in the production of growth factors which, in turn, stimulates tumor survival. The induction of Cx26 and Cx30 in the epidermis adjacent to melanoma putatively leading to GJIC or signaling via hemichannels may play a role in this feedback mechanism by inducing proliferation and other functions. An interruption of this vicious circle may provide a novel therapeutic approach.

9.5 Tight Junctions

In simple epithelia and endothelia Tight Junctions (TJs) are responsible for the formation and maintenance of the tissue barrier between distinct compartments by controlling the paracellular pathway (“barrier function”) (reviewed in Stevenson and Keon 1998; Tsukita et al. 2001). More recently the involvement of TJs in the barrier function of a complex epithelium, the epidermis, was shown (Brandner et al. 2002, 2003; Furuse et al. 2002; Langbein et al. 2002; Pummi et al. 2001). In addition, TJs prevent the diffusion of membrane proteins and lipids from the apical to the basolateral side of an epithelial cell sheet, helping to maintain cell polarity (‘fence function’) (reviewed in Mitic and Anderson 1998; Tsukita et al. 2001). Therefore TJs are crucial for the epithelium to generate chemical and electrical gradients that is necessary for vectorial transport processes such as absorption and secretion (reviewed in Martin and Jiang 2009). Moreover, TJ molecules act as intermediates and transducers in cell signaling, thus playing a role in the processes of polarity, cell differentiation, cell growth, and differentiation. Finally, TJs act as cell–cell adhesion molecules and as a barrier to cell migration (reviewed in Martin and Jiang 2009).

TJs are composed of integral transmembrane proteins (Claudin 1–24, Occludin and junctional adhesion molecules A-C, 4 (JAMs)), peripheral plaque proteins (Zonula occludens (ZO) proteins 1–3, MAGI 1–3, MUPP-1, PAR-3, PAR-6, AF-6, CASK, CAROM) and associated proteins (Symplekin, ZONAB, Cingulin, Rab-13, Rab-3B, c-src, α -catenin, PKA, ZAK, and Rho-GTPases). The molecular composition of TJs is highly complex and varies according to the cell type and degree of differentiation. TJ molecules from neighboring cells associate and form paired strands which seal the paracellular pathway and which contain aqueous pores or paracellular channels, explaining the ion and size selectivity for passing molecules of TJ (Tsukita and Furuse 2000).

In cancer, disruption of TJs should occur in three critical steps: (1) detachment of the tumor cell from the primary tumor, (2) intravasation of the tumor through the endothelium, and (3) extravasation of the circulating tumor cell (reviewed in Martin and Jiang 2009). Early studies have shown a correlation between lack of TJs and tumor differentiation and there is evidence that TJs need to be overcome by cancer cells in order to metastasize (reviewed in Martin and Jiang 2001, 2009). Cancer cells frequently exhibit deficiencies in TJ function, as well as decreased differentiation and cell polarity (Soler et al. 1999; Weinstein et al. 1976). Loss of TJ integrity may be particularly important in allowing the diffusion of nutrients and other factors necessary for the survival and growth of the tumor cells (Mullin et al. 1997). In addition, decreased polarity and differentiation may be important for the metastatic phenotype, where individual cells must leave the primary site and enter the blood vessels to reach distant sites (Ren et al. 1990).

Electron microscopy studies in human thyroid tumors showed that TJs decrease in number and are attenuated during carcinogenesis, which is associated with loss of tumor differentiation (Kerjaschki et al. 1979). Expression of TJ proteins is decreased in some cancer types, e.g., ZO-1 and occludin in gastrointestinal adenocarcinoma (Kimura et al. 1997), occludin in epithelial-derived tumors (Li and MRSny 2000), claudin 3 in

glioblastoma multiforme (Wolburg et al. 2003), claudin 1 in sporadic and hereditary breast cancer (Kramer et al. 2000) and claudin 7 in ductal carcinoma of the breast (Kominsky et al. 2003). On the other hand, some TJ molecules appear to be up-regulated in some cancers. We found protein expression of claudin 3, 4, and 5, occludin and ZO-1 in Merkel cell carcinoma cells (Haass et al. 2003b). Strikingly, expression of some claudin family members is highly elevated in various human cancers, e.g., claudin 7 in two breast cancer cell lines (Nacht et al. 1999), claudin 1 in colorectal cancer (Miwa et al. 2000), claudins 3 and 4 in ovarian (Hough et al. 2001; Rangel et al. 2003) and prostate cancer (Long et al. 2001).

In recent publications the expression of TJ proteins in melanoma tissues and cultured melanoma cells was described on RNA and on protein level (Cohn et al. 2005; Leotlela et al. 2007; Morita et al. 2008; Schmitt et al. 2007; Smalley et al. 2005). In a tissue array study, Claudin-1 was found to be significantly reduced in metastatic melanoma (Cohn et al. 2005). These data were, however, directly contradicted by another study (Leotlela et al. 2007). In this study Claudin-1 appeared to contribute to melanoma cell invasion, as transient transfection of melanoma cells with Claudin-1 increased metalloproteinase 2 (MMP-2) secretion and activation, and subsequently, motility of melanoma cells as demonstrated by wound-healing assays. Conversely, knockdown of CLDN1 by siRNA resulted in the inhibition of motility, as well as decreases in MMP-2 secretion and activation (Leotlela et al. 2007).

In contrast to most cancers, where levels of ZO-1 are typically down-regulated, leading to increased motility, we found that ZO-1 expression is up-regulated in melanoma cells and is located at adherens junctions between melanoma cells and fibroblasts (Smalley et al. 2005). Immunofluorescence and co-immunoprecipitation studies showed co-localization of ZO-1 with N-cadherin. Down-regulation of ZO-1 in melanoma cells through RNA interference produced marked changes in cell morphology – leading to a less dendritic, more rounded phenotype. Consistent with a role in N-cadherin-based adhesion, RNAi-treated melanoma cells were less adherent and invasive when grown in a collagen gel. These data provided the first evidence that increased ZO-1 expression in melanoma contributes to the oncogenic behavior of this tumor and further illustrated that protein products of genes, such as ZO-1, can function in either a pro- or anti-oncogenic manner when expressed in different cellular contexts (Smalley et al. 2005).

In summary, while it appears that functional TJs may be tumor suppressors, the up-regulation of certain TJ proteins can contribute to oncogenic behavior. The relationship between TJ protein overexpression and cancer initiation or progression is thus unclear at present, but may be explained by the lack of functional TJs and that the up-regulated TJ proteins therefore likely function through TJ-independent mechanisms.

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Abstract The pronounced therapy resistance of melanoma remains as an unsolved problem, and induction of apoptosis or sensitization for proapoptotic signals represent suitable targets. Extrinsic (death receptor-mediated) and intrinsic (mitochondria-mediated) proapoptotic pathways have been described, and the better understanding how proapoptotic pathways are blocked in melanoma may provide a basis for the development of new therapeutic strategies.

10.1

Introduction: The Critical Role of Apoptosis Deficiency for Melanoma

Increased incidence of melanoma in the last decades has been described for the white populations worldwide, and the early dissemination in association with a pronounced chemotherapy resistance accounts for an unbroken high mortality (see also Chap. 3). Many clinical trials have been conducted for improving chemotherapy protocols in palliative treatment. Also, biotherapy, including IL-2 and IFN- α as well as different vaccination protocols aiming at an enhancement of the immune response, have been investigated. However, until present, there is no systemic treatment that has significantly extended the survival of patients with disseminated melanoma (Fang et al. 2008; Garbe et al. 2010).

Although multiple cellular mechanisms may be involved in chemoresistance of cancer cells including defects in drug transport and detoxification, the predominant cause of therapy resistance appears to be apoptosis deficiency. Thus, the common target of most anticancer therapies (chemotherapy, biotherapy, radiation) is the elimination of cancer cells by proapoptotic programs (Eberle et al. 2007). Chemotherapeutic drugs cause cellular or DNA damage, which strongly induces cell-intrinsic apoptosis pathways, and cytotoxic T-lymphocytes express death ligands to trigger extrinsic apoptosis pathways in target cells.

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Proapoptotic pathways represent safeguard mechanisms of the organism to prevent tumor growth. Thus, intrinsic apoptosis pathways may be activated in cancer cells by different kinds of cellular dysfunction also including oncogene activation, and the aberrant protein expression of cancer cells may induce an immune response which employs extrinsic proapoptotic signals such as death ligands. The resistance to apoptosis therefore appears as a prerequisite for initiation of cancer growth, and in the second run it also enables a tumor cell to survive chemotherapy treatment.

Melanoma reveals a high chemotherapy resistance and also appears resistant to an immune attack. This may be concluded from the high immunoreactivity, which is evident by spontaneous partial regression frequently seen in initial tumor stages, as well as by often high numbers of tumor-infiltrating lymphocytes (Fig. 10.1). Thus, proapoptotic therapeutic strategies may follow two goals, namely, sensitization for chemotherapy (intrinsic pathways) and sensitization of melanoma cells for an immune response (see also Chap. 17) by activation of extrinsic pathways (Fig. 10.2).

Apoptosis deficiency of cancer cells is mediated by inactivation of proapoptotic or activation of antiapoptotic factors. Both effects may result from an enforcement of survival pathways. In normal tissues, homeostasis is maintained by a balance between cell proliferation and cell death. In this way, programmed cell death (apoptosis) represents an active cellular process, whose physiological endpoint is phagocytosis by macrophages or neighboring cells (Kerr et al. 1972). Due to the key function of apoptosis for the cells fate, the pathways are tightly regulated. Various cellular options for counter-regulation, necessary for the survival of normal cells, abet tumor cells to escape from apoptosis control.

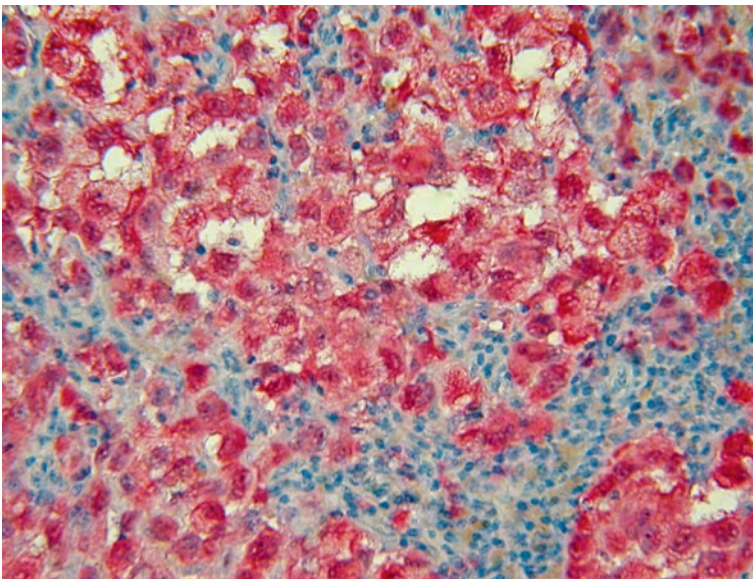


Fig. 10.1 Immunogenicity of malignant melanoma. Pronounced lymphocytic tumor infiltrates are seen in a section of malignant melanoma. Lymphocytes: gray with small nuclei. Melanoma cells: stained red by S100 antibody

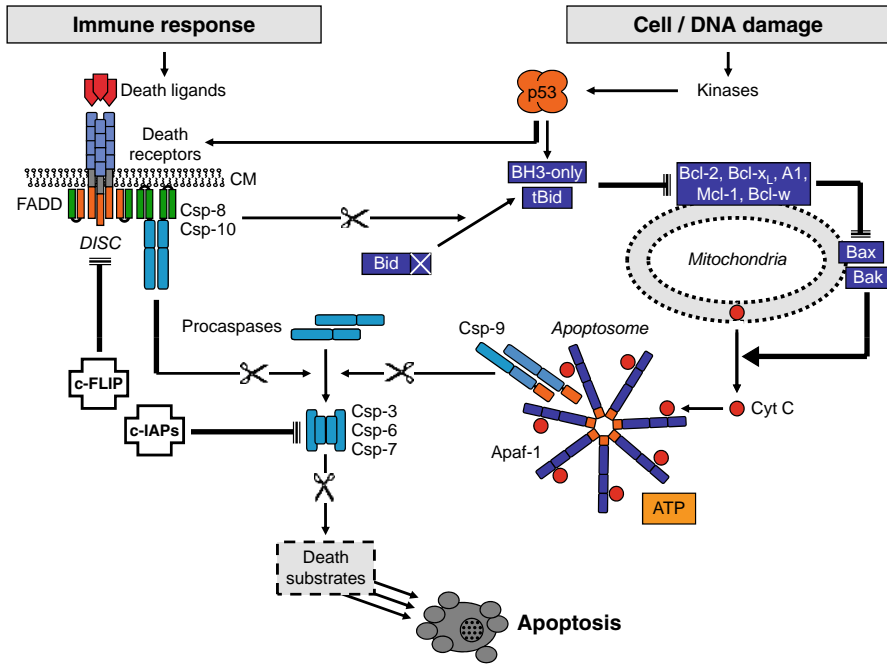


Fig. 10.2 Signaling pathways for regulation of apoptosis. Extrinsic proapoptotic pathways (left) are triggered by death ligands released from immune cells whereas intrinsic proapoptotic pathways (right side) are initiated upon cellular damage via p53 activation. Two multiprotein complexes, namely, the DISC (death-inducing signaling complex) and the apoptosome play particular roles. Caspase 8/10 binding to the DISC is mediated by the adaptor protein FADD and the interaction of death domains (orange) and death effector domains (green), while binding of caspase-9 to the apoptosome is mediated by the caspase recruitment domains (orange). The caspase-8/-10 homolog c-FLIP inhibits activation of the initiator caspases at the DISC, while c-IAPs block effector caspases (-3, -6, -7). FADD Fas-associated death domain, Csp caspase, tBid truncated Bid, APAF-1 apoptotic protease-activating factor-1, ATP adenosine triphosphate, Cyt C cytochrome C, BH3-only BH3-only proteins. c-FLIP cellular homolog of FLICE-inhibitory protein, c-IAPs cellular inhibitor of apoptosis proteins, CM cytoplasmic membrane. A scissors indicates protease activity

10.2 Intrinsic Apoptosis Pathways

Intrinsic pathways are induced by different kinds of cellular stress situations as DNA damage, hypoxia, oncogene activation, or other intrinsic problems. A master regulator in this pathway is the tumor suppressor and transcription factor p53, which is negatively controlled by the ubiquitin ligase HDM-2 but which is stabilized by phosphorylation or acetylation in response to cellular stress. Apoptosis induced by p53 is related to the upregulation of death receptors as well as of proapoptotic Bcl-2 proteins as Bax, Noxa, Puma, Bik/Nbk, and Bid (Zuckerman et al. 2009) (Fig. 10.2).

The large family of pro- and antiapoptotic Bcl-2 proteins critically controls the mitochondrial apoptosis pathway. Bcl-2 proteins are characterized by at least one of four conserved

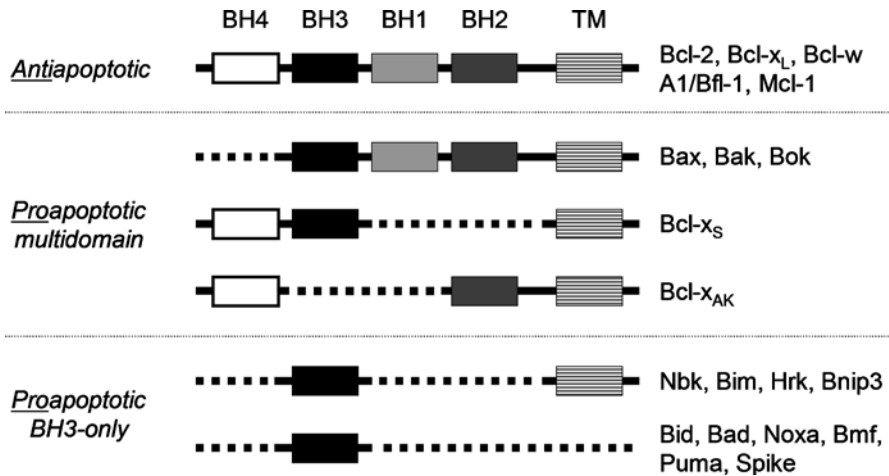


Fig. 10.3 Structure of pro- and antiapoptotic Bcl-2 proteins. A schematic view is given of the principle structures of Bcl-2 proteins. The presence of up to four Bcl-2 homology domains (*BH*) is indicated. Unlike the schematic view, Bcl-2 proteins have variable lengths. Besides the main groups of multidomain antiapoptotic, multi-domain proapoptotic (Bax/Bak group), and BH3-only proteins, two proapoptotic splice variants of Bcl-x with an unusual domain structure that have been investigated in melanoma are shown here. BH3-only proteins may have a transmembrane domain (*TM*) or not

Bcl-2 homology domains (BH1–BH4) (Fig. 10.3). Whereas antiapoptotic proteins as Bcl-2 itself usually share all four domains, proapoptotic proteins subdivide in multidomain proteins with BH1, BH2, and BH3 as Bax and the subfamily of BH3-only proteins (Chipuk et al. 2010). In addition, there are several alternative splice forms. Thus Bcl-x_S and Bcl-x_{AK} are alternative splice forms of the Bcl-x gene, which reveal an atypical structure and proapoptotic functions that have not yet been clearly defined (Hossini and Eberle 2008).

The multidomain proapoptotic proteins Bax and Bak are believed to induce pores in the mitochondrial membrane for release of mitochondrial factors such as cytochrome C. Their proapoptotic potential is however suppressed by antiapoptotic Bcl-2 proteins, which neutralize proapoptotic Bcl-2 family members through heterodimerization, thus protecting the membrane integrity. Finally, BH3-only proteins function as sensors in apoptosis control. Once activated through different kinds of cellular damage, they may bind to antiapoptotic Bcl-2 proteins thus freeing Bax and Bak. Some of them may also activate Bax or Bak directly as described for Bid and Bim (Chipuk et al. 2010).

The proteins Bax and Bak are mutually exchangeable, seen in mice deficient for either one of the two. These mice are viable, whereas the double knock-out strongly impairs developmental apoptosis, resulting in perinatal death. Also cultured cells deficient for both proteins are widely blocked in apoptosis (Lindsten et al. 2000). Deficiency of Bcl-x_L is also lethal, whereas Bcl-2 knock-out mice are viable, but reveal developmental defects particularly concerning increased apoptosis in melanocyte precursors, thus underlining the particular role of Bcl-2 for the melanocytic lineage (Korsmeyer 1999).

The balance between pro- and antiapoptotic Bcl-2 proteins controls the permeability of the mitochondrial membrane. Once a certain threshold is reached, a rapid release of mitochondrial intermembrane factors is induced, which exert specific proapoptotic activities in the cytoplasm as reported for cytochrome C, endonuclease G, AIF (apoptosis-inducing factor), Smac/DIABLO,

and HtrA2/Omi. Thus, cytochrome C released into the cytosol induces formation of the apoptosome, a protein complex consisting of Apaf-1, ATP, cytochrome C, and the initiator caspase-9 (Chipuk et al. 2010). Here, caspase-9 is activated by induced proximity, and it initiates a subsequent caspase signaling cascade. Caspases (aspartate-specific cysteine proteases) represent hallmarks in apoptosis. They are synthesized as inactive zymogens and activate each other by proteolytic processing. Proapoptotic caspases separate in initiator caspases (2, 8, 9, and 10) and effector caspases (3, 6, and 7) (Pop and Salvesen 2009) (Fig. 10.2).

10.3

Extrinsic Apoptotic Pathways

Cytotoxic T-lymphocytes and natural killer cells employ death ligands as TNF- α (tumor necrosis factor), CD95L/FasL, and TRAIL (TNF-related apoptosis-inducing ligand), which trigger extrinsic apoptosis in target cells (Fig. 10.2) (Chan and Housseau 2008). They bind to four death receptors (TNF-R1, CD95, TRAIL-R1/DR4, and TRAIL-R2/DR5), whereas decoy receptors (DcR 1–3 and OPG) do not forward the proapoptotic signal (Fig. 10.4). Characteristic for the proapoptotic program is death receptor oligomerization

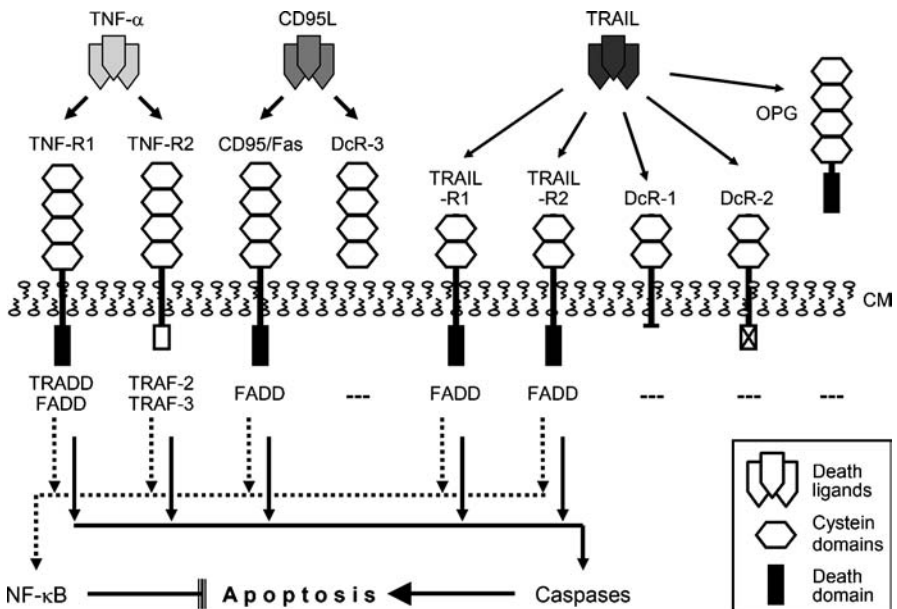


Fig. 10.4 Receptors for death ligands. Receptors of TNF- α , CD95L/FasL, and TRAIL are shown. They enclose four death receptors, indicated by a black death domain, four decoy receptors (DcR, OPG), and TNF-R2. DcR-1 has a truncated domain, DcR-2 has got a nonfunctional death domain, while DcR-3 and OPG are extracellular. TNF-R2 is furnished by a cytoplasmic domain, which does not trigger activation of caspase but of NF- κ B. Two pathways are indicated below, namely, caspase activation and NF- κ B activation. Death receptors can activate both. *FADD* Fas-associated death domain, *TRADD* TNF receptor-associated death domain, *TRAF* TNF receptor-associated factor, *CM* cytoplasmic membrane. Outside the cytoplasmic membrane, the receptors have 2 or 4 cysteine-rich domains (hexagons)

upon binding of the death ligand and formation of a membrane-bound, death-inducing signaling complex (DISC). Here, attached initiator caspases (8 and 10) get activated due to induced proximity or autocatalytic proteolysis. Besides their proapoptotic function, death receptors may also activate NF- κ B and MAPK-related pathways, and NF- κ B is also activated by TNF-R2, which does not trigger caspases (Guicciardi and Gores 2009) (Fig. 10.4). The NF- κ B-mediated transcriptional activation of antiapoptotic proteins as c-FLIP, Bcl- x_L , and c-IAPs may enable a balanced response to death signals (Karin 2006).

The outcome of both extrinsic and intrinsic pathways is the activation of initiator caspases, which in turn promote a caspase cascade leading to active effector caspases. These cleave a large subset of cellular proteins (death substrates) including enzymes for DNA repair and modification as well as signaling and structural proteins, thus completely reprogramming the cell for apoptosis (Fischer et al. 2003). Crosstalk between the pathways may lead to mutual enhancements as transactivation of death receptors by p53 or cleavage and activation of the proapoptotic Bcl-2 protein Bid by caspase-8. Truncated Bid is a BH3-only Bcl-2 protein and involved in activation of the mitochondrial pathway (Fig. 10.2).

10.4

The Ambiguous Role of p53 in Melanoma

In contrast to epithelial tumors, inactivating mutations of p53 are only rare in melanoma. However, p53 found frequently accumulated in the nucleus of melanoma cells seems to exhibit only little transcriptional activity, likely indicative for a post-translational inactivation. Accordingly, melanoma cell lines with wildtype-p53 did not respond to exogenous p53 overexpression, whereas apoptosis was induced in p53-mutated melanoma cells by overexpression of a functional p53 (Satyamoorthy et al. 2000).

The mechanisms involved in p53 inactivation may be related to its negative regulator HDM-2, which is frequently upregulated in melanoma. Its inhibition increased apoptosis in melanoma cells, which were resistant to p53 overexpression (Smalley et al. 2007). Another explanation for p53 inactivation comes from the neuroectodermal marker protein S100B frequently expressed in melanoma and used in melanoma immunohistology. Recent findings suggest that S100B forms a complex with p53 which results in an inhibition of p53 activation, as demonstrated after UVB irradiation (Lin et al. 2010).

Thus, despite the low frequency of p53 mutations, the loss of p53 function may critically contribute to the pronounced chemotherapy resistance of melanoma. The identification of the pathways involved and the overcoming of p53 inactivation is a major challenge in melanoma therapy.

10.5

Role of Caspase Downregulation and Inhibitor of Apoptosis Proteins

The initiator caspases 8 and 10, which are essential for death ligand-induced apoptosis (Fig. 10.2), are frequently downregulated in tumors, which may result from gene silencing by DNA methylation. In melanoma cell lines, both caspases are highly expressed, but they

were downregulated in cell clones selected for TRAIL resistance (Kurbanov et al. 2007). In addition, initiator caspases may be blocked by c-FLIP, which is also frequently expressed in melanoma cells and in metastatic melanoma (Irmeler et al. 1997). Downregulation of c-FLIP also resulted in enhanced sensitivity of melanoma cell lines for TRAIL and CD95-mediated apoptosis (Geserick et al. 2008). Thus, an inhibition early in extrinsic pathways appears as important for melanoma apoptosis resistance.

Many proapoptotic pathways merge at the level of effector caspases (Fig. 10.2), which are suppressed by c-IAPs (Nachmias et al. 2004). In melanoma, expression of survivin, livin/ML-IAP, and XIAP (X-linked IAP) correlated with drug resistance, progression, and survival, and their downregulation increased melanoma cell chemosensitivity *in vitro* and in xenotransplants (Yan et al. 2006). Downregulation of XIAP was also seen after combined treatment of melanoma cells with the kinase inhibitor indirubin and TRAIL (Berger et al. 2010), and incomplete caspase-3 processing seen in response to TRAIL was related to high XIAP expression (Hornle et al. 2010).

Thus, the family of c-IAPs acts at a bottleneck in apoptosis regulation, and efficient apoptosis induction in melanoma cells may also require inhibition of c-IAPs.

10.6

The Role of Bcl-2 Proteins in Melanoma Apoptosis Resistance

The mitochondrial, proapoptotic pathway is critically controlled by the three groups of Bcl-2 proteins (Figs. 10.2 and 10.3). The expression of both antiapoptotic proteins as Bcl-2, Bcl-x_L, and Mcl-1 as well as many proapoptotic proteins as Bax, Bak, Bid, Bad, PUMA, and Noxa has been reported in melanoma cells, whereas some others such as Bcl-x_s and Bik/Nbk were lacking. A high Bcl-2/Bax ratio has been correlated to resistance, and exogenous Bcl-2 overexpression reduced sensitivity of melanoma cells for CD95L, TRAIL, and ceramide (Eberle et al. 2007). Expression of antiapoptotic Bcl-2 proteins has been attributed to survival pathways, as Bcl-2 is controlled by MITF (microphthalmia-associated transcription factor) or Bcl-x_L is controlled by NF-κB (Eberle et al. 2007; Karin 2006).

Despite the significant role of Bcl-2 in melanocyte cell survival, its contribution to chemoresistance of metastasized melanoma remains unclear, because Bcl-2 expression did not correlate with prognosis in primary melanomas, and even reduced expression levels were found in metastases, whereas Bcl-x_L and Mcl-1 were upregulated in metastases (Zhuang et al. 2007). On the other hand, proapoptotic Bcl-2 proteins may be upregulated in course of chemotherapy treatment. Thus, taurolidine-induced apoptosis in melanomas correlated with enhanced Bax and reduced Bcl-2 expression (Sun et al. 2007). Particularly, the expression of proapoptotic Bcl-2 proteins as Bax and Bak appeared as of prognostic value, namely, their downregulation in primary melanomas was correlated with unfavorable prognosis (Fecker et al. 2006).

Thus high expression of antiapoptotic Bcl-2 proteins in melanoma cells may elevate the threshold for an activation of the mitochondrial pathway and thus may also critically contribute to chemotherapy resistance. Strategies should however not only focus on Bcl-2 but the whole family should be considered. Antisense strategies for different antiapoptotic Bcl-2 proteins, overexpression of proapoptotic family members as well as BH3 agonists might be applicable.

10.7

Blockage of Death Receptor–Mediated Pathways in Melanoma

Despite the high immunoreactivity, growing melanomas withstand the proapoptotic attack based on granzymes and death ligands expressed by immune cells (Fig. 10.1). For melanoma-infiltrating lymphocytes, expression of TNF- α , CD95L, and TRAIL has been proven (Thomas and Hersey 1998). This indicates the high selective pressure for melanoma cells to acquire death ligand resistance. Accordingly, molecular changes in the death receptor-mediated pathways are frequently seen. Thus, melanoma cell lines often reveal resistance to CD95 activation, and cultures of metastases were more resistant than those of primary tumors. Loss of CD95 expression and missense mutations in the death domain as well as high Bcl-2 or Mcl-1 expression have been related to resistance to CD95 (Chetoui et al. 2008; Eberle et al. 2007).

Also resistance to TNF- α and loss of TNF-R1 have been reported for melanoma cells, which may be correlated to promoter hypermethylation (Kaminski et al. 2004). In contrast, neither mutations of TRAIL death receptors nor involvement of decoy receptors (DcR-1, DcR-2) have been identified in melanoma cells so far. However, expression of TRAIL-R1, which mediates high TRAIL sensitivity in melanoma cells when expressed, was frequently lost. In contrast, TRAIL-R2 is constitutively expressed but the downstream pathways may be inactive as shown in melanoma cell lines (Kurbanov et al. 2005). Inactivation of TRAIL-R2 may result from its decreased glycosylation or from high c-FLIP expression (Geserick et al. 2008; Wagner et al. 2007).

Whereas no clear correlation was found to the expression of antiapoptotic factors, TRAIL resistance in TRAIL-selected melanoma cells clearly correlated with downregulation of proapoptotic regulators as initiator caspases, DR4 and BH3-only proteins (Kurbanov et al. 2007). Thus, death ligand resistance can be mediated at multiple steps as enclosing death receptors, initiator caspases, and their inhibitor c-FLIP as well as overexpression of antiapoptotic Bcl-2 proteins and downregulation of proapoptotic Bcl-2 proteins.

10.8

Roles of MAP Kinase Pathways in Apoptosis Resistance

Central signaling pathways downstream of growth factor receptors as of MAPKs, PI3K/Akt, and NF- κ B are not only critically implicated in enhanced proliferation of cancer cells but also in their apoptosis resistance (see also Chap. 13). These survival pathways are frequently activated in melanoma. They contribute to the control of a number of apoptosis regulators either by affecting the transcriptional level through regulation of respective transcription factors or by regulating the activity of apoptosis factors directly through phosphorylation (Eberle et al. 2007).

The canonical MAPK pathway via RAF, MEK, and ERK is highly active in melanoma, which is mainly attributed to activating mutations in either *B-Raf* or *N-Ras* (Davies et al. 2002). The MAPK pathway results in activation or control of a multitude of transcription

factors, which may further control the expression of apoptosis regulators. Thus, the transcription factor MITF plays a particular role in apoptosis resistance of melanoma cells due to the MITF-mediated upregulation of Bcl-2 in melanocytic cells as well as due to upregulation of Livin reported for melanoma cells (Dynek et al. 2008). Further transcription factors involved in apoptosis resistance have been identified as of the Ets and CREB/ATF families, which may as well contribute to upregulation of Bcl-2 or Bcl-x_L in course of MAPK activation (Eberle et al. 2007) (see also Chap. 7). Examples for the direct regulation of apoptosis factors are the inactivation of the BH3-only proteins, Bad and Bim, through MAPK phosphorylation (Inamdar et al. 2010).

Improved understanding of the significance of activated survival pathways in melanoma has resulted in the development of selective drugs, which are in clinical trials or preclinical testing (see also Chap. 16). Due to the high frequency of activating mutations, B-RAF represents a most promising target in melanoma (Davies et al. 2002). Its inhibition by siRNA caused growth arrest and promoted apoptosis. Also the inhibitor sorafenib, which targets several kinases including B-RAF, triggered apoptosis in B-RAF-mutated melanoma cells and reduced melanoma growth in mice. However, in clinical trials, sorafenib alone or in combination with chemotherapy so far could not produce sufficient clinical response rates. New hope is given into selective B-RAF inhibitors with high affinity to mutant B-RAF^{V600E} (Inamdar et al. 2010).

Also the targeting of the MAPK pathway downstream of RAF as by MEK inhibitors induced apoptosis in melanoma cells, sensitized melanoma cells for TRAIL and reduced growth of lung metastases in mice (Inamdar et al. 2010). Induction of apoptosis in melanoma cells in response to MEK inhibitors was related to upregulation of BH3-only proteins as of PUMA and Bim, downregulation of Mcl-1 as well as mitochondrial translocation of Bim, and release of Bmf from its association with the cytoskeleton (VanBrocklin et al. 2009; Wang et al. 2007). Thus, the inhibition of the MAPK pathway appears as highly promising and may particularly serve as a suitable option in combination therapies.

10.9

Targeting of the PI3K/Akt/mTOR Pathway

A central role in cell survival has been ascribed to the phosphoinositide 3-kinase (PI3K)/AKT pathway, which is related to increased chemoresistance in many tumors (see also Chap. 16). Akt may directly phosphorylate and inactivate several proapoptotic proteins such as Bax, Bad, and caspase-9, and may further inactivate proapoptotic transcription factors such as FoxO and p53. In addition, AKT is linked to the survival pathway of mTOR (mammalian target of rapamycin), which inhibits cell death pathways as apoptosis and autophagy (Stiles 2009).

Melanomas reveal high immunoreactivity for activated, phosphorylated AKT, which may partly be related to mutations of its inhibitor PTEN (phosphatase and tensin homolog on chromosome 10). Antiapoptotic activities of AKT have been described for melanoma cells, such as inactivation of Bad and activation of IKK (I- κ B kinase) leading to a cross-activation of the NF- κ B pathway. The critical role of the AKT survival

pathway in melanoma was demonstrated by expression of a dominant negative AKT mutant, by its siRNA downregulation as well as by overexpression of PTEN, which all triggered apoptosis (Robertson 2005).

By using small molecule approaches, inhibition of PI3K reduced melanoma growth in mice, particularly in combination with inhibition of MEK (Inamdar et al. 2010), and both AKT and mTOR inhibition consistently enhanced apoptosis and chemosensitivity of melanoma cells (Sinnberg et al. 2009). Also, the farnesyl transferase inhibitor lonafarnib, which inhibits mTOR signaling, enhanced sorafenib-induced apoptosis in melanoma cells, which was related to induction of ER stress and downregulation of Mcl-1 (Niessner et al. 2010). However, inhibitors of AKT as well as of mTOR did not result in significant clinical responses in metastatic melanoma patients, when used as monotherapy. (Eberle et al. 2007). Although the inhibition of these pathways may not be sufficient by itself, combination strategies may be an option.

10.10

Roles of NF- κ B in Death Ligand and Chemotherapy Resistance

Transcription factors of the NF- κ B family, which function as hetero- or homodimers, are sequestered in the cytoplasm by binding to the inhibitors of NF- κ B (I- κ Bs). Activation and nuclear translocation of NF- κ B via the canonical pathway encloses activation of the I- κ B kinase complex (IKK) and phosphorylation of I- κ B for targeting its proteasomal degradation. Constitutive activation of NF- κ B is a frequent hallmark of various tumors. Its anti-apoptotic activities result from transactivation of antiapoptotic factors such as c-IAP proteins, c-FLIP, and Bcl-x_L (Karin 2006).

Constitutive NF- κ B activation has also been reported for melanoma, and high NF- κ B activities in melanoma cells had been correlated with resistance to radio- and chemotherapy. NF- κ B may also be induced by death ligands, and on the other hand it may decrease death ligand-induced apoptosis, forming a negative feedback loop. There is considerable hope to enhance TRAIL sensitivity by inhibition of NF- κ B, as reported in colon carcinoma cells. Also for melanoma cells, a relation between NF- κ B suppression and enhanced TRAIL sensitivity had been suggested (Plantivaux et al. 2009). However, there was no clear correlation between TRAIL sensitivity and basic NF- κ B activities in melanoma cells, and DR4-positive melanoma cell lines were highly TRAIL-sensitive, despite a strong NF- κ B activation upon TRAIL treatment (Kurbanov et al. 2007). Thus, the particular role of NF- κ B for death ligand sensitivity remains to be further unraveled.

The targeting of NF- κ B by IKK knockdown as well as by an IKK β inhibitor resulted in induced apoptosis *in vitro* and reduced melanoma growth in mouse models (Yang et al. 2007). NF- κ B may also be downregulated by proteasome inhibitors, which prevent the breakdown of I- κ B. Thus treatment with the proteasome inhibitor bortezomib resulted in effective growth inhibition and induction of apoptosis in melanoma cells *in vitro* and it reduced melanoma growth in mouse models. However, in a phase I trial in patients with advanced melanoma using bortezomib in combination with temozolomide, the clinical outcome was not clearly correlated to an inhibition of NF- κ B (Su et al. 2010). Thus,

inhibition of NF- κ B was not sufficient, when used as monotherapy but its value in combinations has still to be investigated in more detail.

Extensive crosstalk of different survival pathways may further lead to their mutual enhancement. Thus NF- κ B is linked with the MAPK and PI3K/Akt pathways, and simultaneous inactivation of several pathways may be necessary for obtaining a sufficient effect. As an example, the proapoptotic BH3-only protein Bad can be inactivated by phosphorylation through both the MAPK and Akt pathway. Thus, Bad inactivation could be efficiently prevented in melanoma cells only by blocking of both pathways (She et al. 2005).

10.11

The Promise of New Proapoptotic Strategies: BH3 Mimetics

Due to the results of clinical trials performed in melanoma so far, the combination of new therapeutic strategies appears as necessary, and the combination of survival pathway inhibitors with proapoptotic strategies is most promising. Frequently, chemotherapeutics have been used in combinations, which cause cellular stress situations as DNA damage and trigger apoptosis via intrinsic pathways. A selective induction of apoptosis in cancer cells may however be more efficient and better tolerated.

When considering the important role of the mitochondrial pathway in melanoma, approaches targeting the Bcl-2 protein family appear of particular interest. Thus Bcl-2 antisense strategies have been applied, which revealed *in vitro* and in mouse models significantly induced apoptosis and sensitization for chemotherapy. However, in clinical trials, the combination of Bcl-2 antisense with chemotherapy was not sufficiently effective so far (Bedikian et al. 2006). This may be related to the finding that expression of Bcl-2 may even be reduced in metastatic melanoma (Zhuang et al. 2007).

Due to the possible mutual substitution of antiapoptotic Bcl-2 proteins in melanoma, the simultaneous targeting of several proteins may be necessary. As an alternative strategy appears the overexpression of proapoptotic Bcl-2 proteins. In several studies, it was demonstrated that overexpression of proapoptotic Bcl-2 proteins efficiently induced apoptosis in melanoma cells, enhanced chemosensitivity and decreased melanoma growth in mouse models, shown for Bax, Bik/NBK, Bcl-x_s, and Bcl-x_{AK} (Eberle et al. 2007).

Of particular interest are some new drug developments, which mimic the BH3 domain of proapoptotic Bcl-2 proteins. BH3 is required for the interaction between proapoptotic Bcl-2 proteins and the hydrophobic pocket of antiapoptotic Bcl-2 proteins formed by BH1, BH2, and BH3. The BH3 mimetics are peptides or small molecules structurally related to different BH3 domains, and depending on their structure, they have the potential to block different antiapoptotic Bcl-2 proteins (Adams and Cory 2007).

There are several preclinical reports on the effects of BH3 mimetics in melanoma. Thus the natural BH3 mimetic gossypol, which is a compound enclosed in cotton seeds, induced cell death in melanoma cells with even higher efficacy than some chemotherapeutics (Shelley et al. 1999). Apoptosis resistance due to high levels of anti-apoptotic Bcl-2 proteins was overcome by the BH3 mimetic TW-37 when used in combination with the MEK inhibitor U0126 (Verhaegen et al. 2006). A synergistic induction of mitochondrial

apoptosis has been reported in melanoma cells by combination of the BH3 mimetic ABT-737 and the proteasome inhibitor MG-132, which resulted in simultaneous upregulation of Noxa (Miller et al. 2009). Efficient induction of apoptosis has also been described for the combination of ABT-737 and a Mcl-1 knockdown (Keuling et al. 2009). A combination of obatoclast, a BH3 mimetic with inhibitory activity against Mcl-1, with an ER stress-inducing compound resulted in marked induction of apoptosis in melanoma cells, which was dependent on Mcl-1 inhibition and again induction of Noxa (Jiang et al. 2009).

New BH3 mimetics designed by computer-based modeling are presently developed and more effective ones, preferentially targeting several antiapoptotic Bcl-2 proteins, may be expected in future. Considering the high dependency of apoptosis induction in melanoma cells on the mitochondrial pathway, BH3 mimetics provide hope for an efficient targeting of melanoma. The complex mutual regulation of Bcl-2 proteins, however, needs further clarification in melanoma to optimize these approaches.

10.12

Death Ligands

Due to the high immunoreactivity of melanoma (Fig. 10.1) but its frequent resistance to death ligands, strategies for enhancing death ligand sensitivity and overcoming resistance appear promising. In contrast to chemotherapy, death ligands largely trigger apoptosis independently of p53 and thus may overcome drug resistance related to p53 inactivation. CD95 agonistic antibodies and TNF- α have shown high efficiency in eradication of xenotransplants in mouse models. Unfortunately, their application in patients is largely prevented due to systemic inflammation (TNF- α) and liver toxicity (CD95L), respectively (Kelley and Ashkenazi 2004; Trauth et al. 1989). Selective expression or induction of these ligands in gene therapeutic approaches however remains an alternative strategy. Thus, expression of CD95L prevented growth of melanoma xenotransplants in mice (Eberle et al. 2003), and its selective expression via conditional replication-competent adenoviral vectors appeared as an efficient approach for killing melanoma cells (Fecker et al. 2010).

As compared to TNF- α and CD95L, systemic application of TRAIL is much better tolerated. Induction of apoptosis and eradication of xenotransplants were demonstrated in a variety of tumor models, whereas toxicity for normal cells remained at a low level. Both TRAIL receptors (DR4 and DR5) may be targeted by recombinant TRAIL or by selective agonistic antibodies, which showed efficiency and synergistic effects with chemotherapy in preclinical studies. TRAIL also demonstrated antitumor activities in clinical trials; it appeared however not as efficient enough when used as monotherapy, as recently shown in a large phase III trial with agonistic antibodies for DR4 for therapy of patients with refractory colorectal cancer (Newsom-Davis et al. 2009; Trarbach et al. 2010).

Melanoma cell lines, which express TRAIL-R1, revealed pronounced sensitivity to TRAIL. Importantly, the majority of primary melanomas were also proven by immunohistology as DR4-positive (Kurbanov et al. 2005). However, melanoma cells responded to TRAIL treatment with an inducible resistance that was correlated with the downregulation of DR4 and initiator caspases (Kurbanov et al. 2007). Chemotherapy, which may upregulate death receptors (Singh et al. 2003), proteasome inhibitors, which may prevent

death receptor degradation (Kurbanov et al. 2007) or IFN- γ , which may upregulate initiator caspases (Fulda and Debatin 2006), may thus be helpful in countering TRAIL resistance.

Indeed, in melanoma cells, the combination with chemotherapeutics revealed enhanced TRAIL sensitivity (Fecker et al. 2011). Sensitization to TRAIL-induced apoptosis was also achieved by combination with an indirubin derivative, which acts as multi-kinase inhibitor (Berger et al. 2011), or by knockdown of Cystatin B, which was related to an inhibition of proteasomal degradation of c-FLIP (Yang et al. 2010). Sensitization of melanoma cells was also seen in combination with resveratrol, which decreased STAT3 and NF- κ B activation and activated a proapoptotic JNK pathway to finally suppress expression of c-FLIP and Bcl-x_L (Ivanov et al. 2008).

Thus, TRAIL appears of high value for targeting melanoma. Interestingly, TRAIL sensitivity may be enhanced by multiple different strategies. It appears as a challenge to identify common principles of death ligand sensitization in future to optimize these strategies.

10.13

Conclusions

The pronounced chemoresistance of melanoma is largely explained by an apoptosis deficiency substantiated by multiple molecular changes. In addition, there is a pronounced resistance to tumor-infiltrating cytotoxic T-lymphocytes, which appears as related to permanent as well as to inducible resistance to death ligands. New treatment strategies are still needed, and combinations of direct inducers of apoptosis with inhibitors of survival pathways may be particularly promising. There is reasonable hope, that nonselective chemotherapies can be substituted by more selective proapoptotic signaling therapies.

Bcl-2 proteins as master regulators of the mitochondrial pathway appear as suitable targets, and small molecule BH3 mimetics may develop into efficient strategies. Finally, TRAIL and, particularly, DR4-mediated approaches as agonistic antibodies, which have so far been underestimated with regard to their potential for melanoma, may be promising candidates. Given the currently fast accelerating developments in cancer research with a steadily increasing number of new drugs, different active drug combinations may be identified in the near future. Finally, due to a heterogeneous pattern of regulation of survival and proapoptotic pathways in melanoma cells, a detailed stratification of melanoma patients on the basis of molecular markers will become essential.

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Abstract Cellular senescence is a program initiated by many stress signals including aberrant activation of oncogenes, DNA damage, oxidative lesions and telomere attrition. Once engaged senescence irreversibly limits cellular proliferation and can potentially prevent tumour formation *in vivo*. The precise mechanisms driving senescence are still not completely defined, although the pRb and p53 tumour suppressor pathways are critical effectors. Senescent cells also develop aberrant gene expression profiles and acquire pro-inflammatory behaviour that may contribute to organismal ageing and age-related diseases, including cancer. It is not yet clear whether the pro-ageing actions of senescent cells can be minimised *in vivo*, but the therapeutic potential of this stress-induced program may depend on establishing a new equilibrium that favours tumour suppressor activity.

11.1 Introduction

Cellular senescence is regarded as an intrinsic stress response mechanism that limits the proliferative lifespan of cells. Senescence is induced by various signals, including telomere attrition (a response often referred to as replicative senescence), activated oncogenes (a process known as oncogene-induced senescence), DNA damage, oxidative lesions and suboptimal culture conditions (reviewed in Collado and Serrano 2006). Irrespective of the initiating trigger, the hallmark of cellular senescence is permanent proliferative arrest, and whereas quiescent cells can be stimulated to resume proliferation, senescence cells cease to respond to mitogenic stimuli.

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The limited proliferative capacity of normal human cells was recognised by Hayflick and Moorehead nearly five decades ago (Hayflick and Moorhead 1961). They reported that primary human fibroblasts ceased proliferating after serial cultivation *in vitro* and although these arrested fibroblasts remained metabolically active for many weeks, they did not initiate DNA replication despite adequate culture conditions. Replicative senescence has since been described in many normal somatic human cells, including epidermal keratinocytes and melanocytes as well as in cells derived from rodents, birds and several other species (Campisi 2001; Kim et al. 2002). The Hayflick limit is often used to refer to the maximum number of population doublings for any given cell population.

11.2

Characteristics of Senescent Cells

Senescent cells have been identified both *in vitro* and *in vivo* using a series of phenotypic features and markers that are not exclusive to the senescent state but act as powerful predictors of senescence when used in combination (reviewed in Campisi and d'Adda di Fagagna 2007; Collado and Serrano 2006). Increased activity of acidic β -galactosidase, termed senescence-associated β -galactosidase (SA- β -gal) is the most widely accepted marker of senescent cells (Dimri et al. 1995). The expression of this enzyme correlates strongly with the senescence state (Fig. 11.1), although it can also be induced by stresses such as serum withdrawal and prolonged cell culture (Severino et al. 2000). SA- β -gal activity derives from residual lysosomal β -galactosidase activity at the suboptimal pH 6.0 (pH 4.5 is optimal) and reflects the increased lysosomal content of senescent cells (Kurz et al. 2000; Lee et al. 2006). More recently, the appearance of DAPI-stained heterochromatic regions, known as senescence-associated heterochromatic foci (SAHF) (Fig. 11.1), which promote the stable repression of certain E2F target genes involved in proliferation, are associated with senescence (Narita et al. 2003). Each SAHF contains portions of a single condensed chromosome, which is enriched for common markers of heterochromatin, including heterochromatin protein 1 γ , histone H3 methylated at lysine 9 (H3K9Me) and the non-histone chromatin protein, HMGA2 (Fig. 11.1) (Narita et al. 2003; reviewed in Adams 2007).

Several other markers of senescence have also been described and validated, including accumulation of the cyclin-dependent kinase (CDK) inhibitors p16^{INK4a}, p15^{INK4b}, an anti-apoptotic bcl-2 member Mcl-1 and the transcription factor Dec1 (Collado and Serrano 2005). Levels of the p53 transcription target and CDK inhibitor p21^{Waf1}, the plasminogen activator inhibitor-1 (PAI-1) protein and miR-34 family of microRNAs are also elevated in senescent cells (Goldstein et al. 1994; He et al. 2007; Wong and Riabowol 1996). Finally, morphological changes such as cell enlargement with a concomitant increase in nuclear size, vacuolisation and cell flattening are typical of senescent cells *in vitro*.

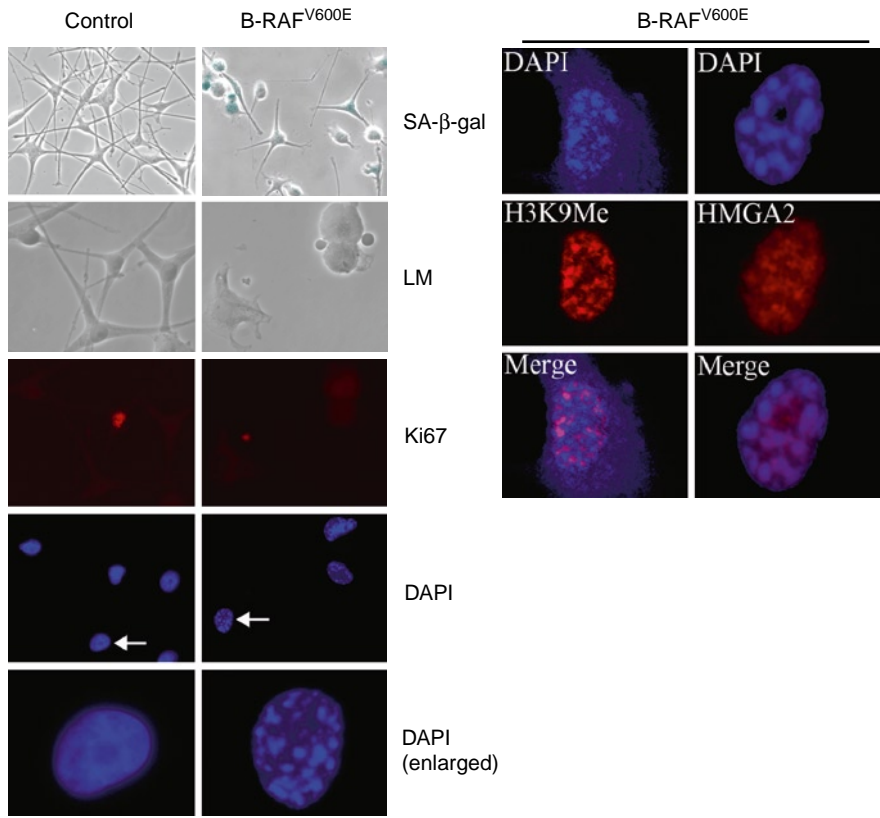


Fig. 11.1 Senescence is associated with positive SA-β-gal activity and appearance of condensed chromatin. B-RAF^{V600E}-induced senescence in human melanocytes is characterised by rapid cell cycle arrest (reduced Ki67), appearance of SAHF (DAPI foci) and increased SA-β-gal activity (*left panel*). Cells enlarged to show DAPI-stained SAHF foci are indicated with arrows. SAHF are enriched for markers of heterochromatin, including H3K9Me and HMGA2 (*right panels*)

11.3 Senescent Cells *In Vivo*

The existence of senescence cells *in vivo* has important implications for multicellular organisms; the onset of stress-induced premature senescence may prevent the development of malignant cancer but the accumulation of damaged senescent cells may eventually compromise tissue integrity and prove detrimental.

11.3.1

Cellular Senescence and Cancer

The observation that aberrant activity of many oncogenes, including RAS, c-MYC and B-RAF trigger senescence *in vitro* (reviewed in Gorgoulis and Halazonetis 2010) suggested that oncogene-induced senescence may act as a barrier to tumourigenesis. Compelling evidence for the tumour suppressor role of oncogene-induced senescence stems from studies in mouse cancer models and human tumours. For instance, pre-malignant lesions in the lung (which developed in a conditional knock-in mouse model expressing oncogenic K-RAS^{G12D}) contained many cells expressing markers of oncogene-induced senescence including p16^{INK4a}, p15^{INK4b}, SAHF and SA- β -gal, whereas lung adenocarcinomas were almost completely devoid of cells positive for these markers (Collado and Serrano 2005). Similarly, constitutively active N-RAS^{G12D} prevented lymphomagenesis by triggering potent senescence in murine lymphocytes (Braig et al. 2005) and H-RAS^{G12V} triggered a dose-dependent senescence response in mammary epithelia (Sarkisian et al. 2007). Further, inactivation of the PTEN tumour suppressor, which acts as a phosphoinositide 3-kinase (PI3K) pathway inhibitor in mouse prostate triggered an acute senescence response that suppressed the development of invasive adenocarcinoma (Chen et al. 2005).

Senescence markers are also abundant in human pre-malignant lesions of the skin, colon, prostate and nervous system, whereas they are almost completely absent in malignant tumours (Bartkova et al. 2006; Chen et al. 2005; Courtois-Cox et al. 2006; Kuilman et al. 2008; Michaloglou et al. 2005). Perhaps the most compelling data come from studies with human naevi (moles), which are small benign tumours of melanocytes that frequently harbour oncogenic mutations in the B-RAF kinase (Pollock et al. 2003) (see also Chap. 7). Naevi remain growth arrested for decades and rarely become melanomas (Kuwata et al. 1993; Maldonado et al. 2004), presumably because aberrant B-RAF signalling induces potent senescence (Dankort et al. 2009; Dhomen et al. 2009; Goel et al. 2009; Gray-Schopfer et al. 2006; Michaloglou et al. 2005). Human naevi display many features of oncogene-induced senescence, including intact telomeres (the repetitive sequences at the ends of each chromosome), increased p16^{INK4a} expression and positive SA- β -gal activity (Michaloglou et al. 2005; Miracco et al. 2002; Mooi and Peeper 2006), although the expression of SA- β -gal in human naevus cells *in vivo* remains controversial (Cotter et al. 2007, 2008; Michaloglou et al. 2008). Intriguingly, recent studies indicate that mutant B-RAF is not present in every cell within a naevus, suggesting that oncogenic B-RAF may not initiate melanocytic hyperplasia (Ichii-Nakato et al. 2006; Lin et al. 2009). This does not discount the possibility that B-RAF triggers senescence, however, as the senescent signal may be transmissible to surrounding cells in a paracrine fashion (see below) (Kuilman and Peeper 2009).

11.3.2

Cellular Senescence and Ageing

Evidence that replicative (telomere-associated) senescence reflects organismal ageing and may contribute to age-related decrements in tissue structure and function is more contentious. Certainly, telomeres progressively shorten with each cell division and DNA damage

foci marking telomere dysfunction increase with age to approximately 20% in very old primates (Herbig et al. 2006). Further, cells with a senescent phenotype are prevalent at sites of age-related diseases, such as osteoarthritis and atherosclerosis (Chang and Harley 1995; Price et al. 2002) and mice lacking the mitotic regulator BubR1 or the p53 homologue, p63, developed age-related pathologies that were associated with cellular senescence (Baker et al. 2004; Keyes et al. 2005).

SAHF-positive senescent fibroblasts also increase with age in primate skin (Herbig et al. 2006) and SA- β -gal positive senescent cells were found to accumulate in the skin of elderly people (Dekker et al. 2009; Dimri et al. 1995) although this latter result was not reproduced (Severino et al. 2000). Conversely, in some 13 studies involving 79 patients with accelerated ageing disorders, such as Werner and Hutchinson-Gilford syndromes, the replicative capacity of fibroblasts were consistently lower than fibroblasts derived from age-matched controls (reviewed in Davis et al. 2007; Maier and Westendorp 2009). Early reports described a weak inverse relationship between replicative lifespan of fibroblasts *in vitro* and the chronological age of the donor, although there was large variability in the data and recent studies using larger cohorts have found no significant association between age and replicative lifespan (Cristofalo et al. 1998; reviewed in Maier and Westendorp 2009). It is worth considering that the majority of these studies utilise mass cell cultures, so that the Hayflick limit, which is comparable in young and older individuals, reflects the cells with the longest proliferative capacity. This does not preclude the possibility that overall replicative capacity declines with age, in fact the replicative lifespan of individual human fibroblast clones were related to donor age (McCarron et al. 1987).

The accumulation of senescent cells may contribute to the ageing process by depleting the regenerative potential of stem cells or by altering tissue structure and function. Many studies suggest that the expression of p16^{INK4a}, which increases during senescence and with age in most rodent, baboon and human tissue tested (Edwards et al. 2007; Herbig et al. 2006; Krishnamurthy et al. 2004; Liu et al. 2009; Melk et al. 2003; Zindy et al. 1997), limits the proliferative capacity of self-renewing stem cells and thus impairs tissue regeneration. Mice lacking p16^{INK4a} retain stem cell division and tissue regeneration with advancing age (Janzen et al. 2006; Krishnamurthy et al. 2006; Molofsky et al. 2006; Signer et al. 2008). In contrast, p16^{INK4a} inhibits the reprogramming of differentiated human cells into induced pluripotent stem cells. Reprogramming can be likened to a rejuvenation process and there is an age-related decline in reprogramming efficiency that can be reversed by inactivation of the p16^{INK4a} gene (Banito et al. 2009; Li et al. 2009).

In spite of the substantial evidence linking p16^{INK4a} expression with senescence and molecular ageing in humans, a recent study suggested that p16^{INK4a} and its homologue p15^{INK4b} may have anti-ageing functions. This work was based on a mouse model genetically engineered to carry two copies of the *INK4a/ARF* locus, which encodes the p16^{INK4a}, p19ARF and p15^{INK4b} tumour suppressor proteins. These mice were less susceptible to cancer, showed increased median longevity and a lower incidence of ageing-associated kidney lesions and DNA damage response (Matheu et al. 2009). Although, the additional p15^{INK4b} and p19ARF genes complicate interpretation of these data it is conceivable that the role of p16^{INK4a} in ageing reflects its expression. The progressive upregulation of p16^{INK4a} may delay ageing by reducing cell proliferation and maintaining stem cell reserves. In contrast, the acute, mitogen-driven upregulation of p16^{INK4a} may promote ageing by

initiating senescence and permanently preventing stem cell proliferation and tissue regeneration.

Senescent cells may also contribute to ageing by altering tissue structure and function; senescent cells secrete many extracellular matrix associated factors and inflammatory proteins. Many of these secreted factors, including TGF β , IL-6 and PAI1, accumulate with ageing. Some, including PAI1 and matrix metalloproteinases, may damage or alter tissue integrity (reviewed in Campisi and d'Adda di Fagagna 2007; Kuilman and Peeper 2009).

Taken together, these findings suggest that irreversibly growth-arrested senescent cells can act as a barrier against tumour formation in young organisms, but their net accumulation may reach a point that compromises tissue function and stem cell renewal leading to the development of deleterious phenotypes with age (Campisi and d'Adda di Fagagna 2007).

11.4

Critical Pathways in Cellular Senescence

Although diverse stimuli can induce a senescence response, they appear to converge on two pathways that initiate and maintain this program. These pathways are regulated by the tumour suppressor proteins p53 and the retinoblastoma protein (pRb), both of which are frequently lost in human cancer cells (Sherr 1996). Importantly, although cancer cells have partially lost the capacity to initiate senescence, the senescence response can be re-engaged by restoring the p53 and pRb pathways, and tumour regression through senescence may be achieved. It has been shown, for instance, that re-instating p16^{INK4a} in human tumour cells resulted in the establishment and maintenance of a senescence response (Haferkamp et al. 2008; Sugrue et al. 1997).

The senescent states induced by the p53 and pRb pathways may be distinct and whether cells engage one or the other pathway appears to reflect the type of stress signal, the tissue and species of origin. Recent data also reveal senescence-inducing pathways that appear to be independent of the p53- and pRb-pathways. For instance, neither p53 nor p16^{INK4a} was required for H-RAS-induced senescence in melanocytes, and senescence induced by B-RAF^{V600E} or N-RAS^{Q61R} did not depend on p16^{INK4a} or p53 (Denoyelle et al. 2006; Zhuang et al. 2008).

11.4.1

The p53 Pathway and Senescence

p53 engages a formidable proliferative arrest by transactivating genes, such as p21^{Waf1} and the miR-34 class of microRNAs (Brown et al. 1997; He et al. 2007; Smith et al. 1996) primarily in response to DNA-damage checkpoint signals triggered by telomere dysfunction and activated oncogenes (Bartkova et al. 2006; Di Micco et al. 2006; Herbig et al. 2004; Ramirez et al. 2001) (see also Chaps. 6 and 5). Shortened dysfunctional telomeres are recognised as DNA double-strand breaks and lead to the activation of the DNA damage

checkpoint (d'Adda di Fagagna et al. 2003). Likewise, oncogene-induced senescence, which causes DNA replication stress including DNA double-strand breaks, triggers a DNA damage response (Bartkova et al. 2006). Consequently, dampening the DNA damage checkpoint via the inactivation of p53-regulators (including ataxia telangiectasia mutated (ATM) and checkpoint-2 (Chk2) kinases) or p53 itself can overcome oncogene-induced and telomere-dependent senescence in human fibroblasts (d'Adda di Fagagna et al. 2003; Di Micco et al. 2006). Similarly, inactivation of the upstream p53 activator, ARF, overcame oncogene-induced senescence in mouse embryo fibroblasts (MEFs) (Kamijo et al. 1997; Serrano et al. 1996), while the loss of p21^{Waf1} caused cells to bypass telomere-dependent replicative and oncogene-induced senescence in normal human fibroblasts and MEFs, respectively (Brown et al. 1997; Pantoja and Serrano 1999; Wei and Sedivy 1999).

Despite the substantial evidence that p53 promotes senescence, recent data indicate that physiological p53 signalling promotes longevity and favours quiescence over senescence (Demidenko et al. 2010; Matheu et al. 2007, 2008). Mice engineered to express mildly elevated levels of wild type p53 display strong resistance to tumourigenesis, normal longevity but decreased levels of ageing-associated damage (Garcia-Cao et al. 2002, 2006). In contrast, mice expressing constitutively active forms of p53 showed accelerated ageing and an increase in the proportion of senescent cells *in vivo* (Dumble et al. 2004; Hinkal et al. 2009). It is likely that the cellular context of the p53 response dictates whether quiescence or senescence is triggered. For instance, inhibition of the growth-promoting mTOR pathway by p53 favours quiescence, whereas the simultaneous activation of p53 signalling along with mTOR (i.e. via oncogenic RAS, loss of PTEN) promotes senescence (Fig. 11.2) (Alimonti et al. 2010; Galluzzi et al. 2010; Korotchkina et al. 2010).

11.4.2

The p16^{INK4a}/pRb Pathway

Although inactivation of the p53 pathway can reverse the senescence arrest in some cells, there is an emerging consensus that this reversal is blocked in cells with an activated p16^{INK4a}/pRb pathway (Beausejour et al. 2003; Herbig et al. 2004; Sakamoto et al. 1993). Active, hypophosphorylated pRb interacts with E2F transcription factors and facilitates chromosome condensation at E2F target promoters. The reorganisation of chromatin leads to the formation of SAHF and the stable repression of proliferation-promoting genes, resulting in the irreversible growth arrest associated with senescence (Narita et al. 2003).

p16^{INK4a} is a positive regulator of pRb and is crucial in generating SAHFs (Narita et al. 2003). Not surprisingly, p16^{INK4a} also acts as a tumour suppressor and is frequently inactivated in established human tumours, and inactivating melanoma-associated mutations in p16^{INK4a} are inherited in melanoma-dense kindreds (Goldstein et al. 2006). In fact, p16^{INK4a}-deficient human melanocytes, derived from melanoma affected individuals, show an extended lifespan and can be immortalised by ectopic expression of telomerase reverse transcriptase, whereas normal melanocytes display neither of these features (Bennett 2003; Sviderskaya et al. 2003). Furthermore, replicative and oncogene-induced senescence are accompanied by accumulation of p16^{INK4a} in primary human cells (Alcorta et al. 1996; Hara et al. 1996; Serrano et al. 1997), while ectopically expressed p16^{INK4a} initiates a

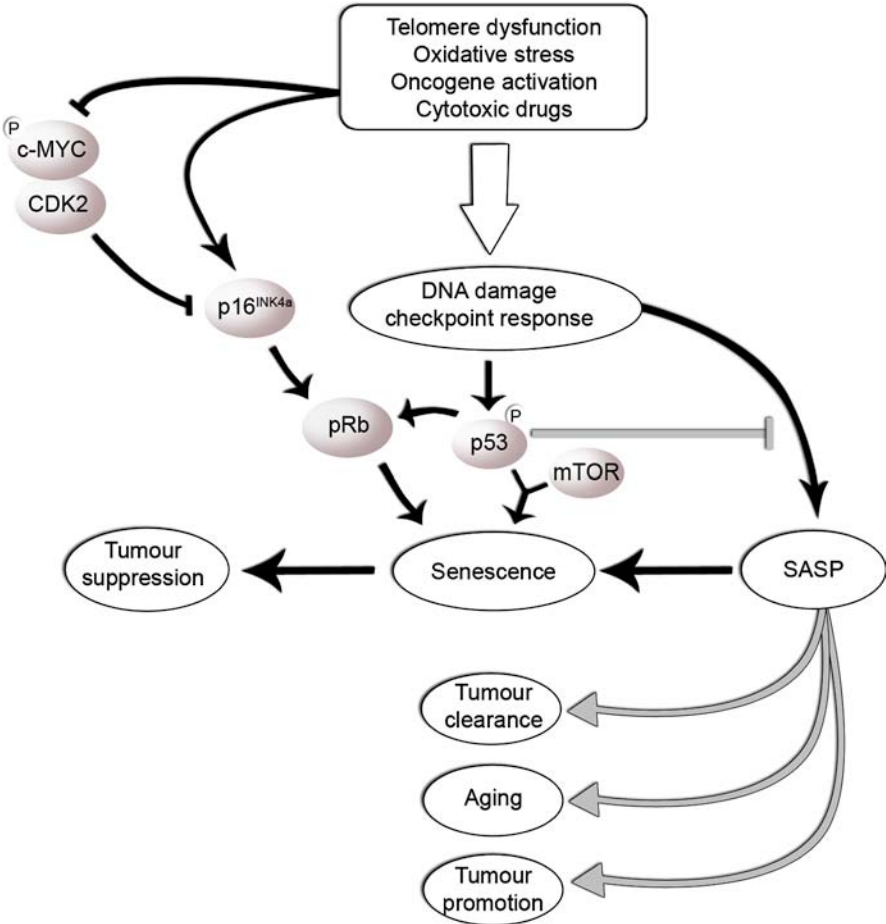


Fig. 11.2 Diverse stress stimuli activate cellular senescence via the DNA damage checkpoint response. Persistent DNA damage leads to the activation of the p53-signalling cascade, which favours senescence when the mTOR growth-promoting pathway is switched on. p53 also signals through the pRb tumour suppressor pathway via the transcriptional induction of the CDK inhibitor p21^{Waf1} (not shown). pRb activation is reinforced by the CDK inhibitor, p16^{INK4a}, which is induced by oncogenic signalling, in part by inhibition of c-MYC-CDK2 transcription activity. Sustained damaged to DNA also promotes the secretion of a large number of proteins, a phenomenon known as the senescence-associated secretory phenotype (SASP). SASP can have different effects on cancer and ageing; it can suppress cancer by reinforcing the senescent state and inhibiting mitogenic signals, it can activate the innate immune response leading to improved clearance of tumour cells, it can impair the function of stem cells and promote ageing, and it could stimulate the proliferation of neighbouring tumour cells

senescence programme characterised by cell cycle arrest, senescence-associated changes in cell morphology, increased SA- β -gal activity and the appearance of SAHF (Dai and Enders 2000; Haferkamp et al. 2008).

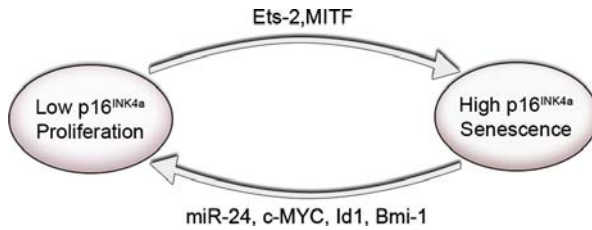


Fig. 11.3 Acute activation of the CDK inhibitor $p16^{\text{INK4a}}$ favours senescence. Aberrant oncogenic activity promotes $p16^{\text{INK4a}}$ accumulation by suppressing the MYC, Id1 and Bmi-1 transcription factors, while inducing the accumulation of the Ets-2 and MITF transcription regulators. As cells near replicative senescence the $p16^{\text{INK4a}}$ -inhibitory microRNA, miR24, is depleted and $p16^{\text{INK4a}}$ translation is restored

$p16^{\text{INK4a}}$ expression is maintained at low levels prior to senescence by miR-24, a negative-regulator microRNA that suppresses $p16^{\text{INK4a}}$ translation. As cells near senescence, miR-24 expression diminishes and $p16^{\text{INK4a}}$ protein accumulates (Lal et al. 2008). Oncogenic signalling also induces $p16^{\text{INK4a}}$ expression by reducing the levels of the $p16^{\text{INK4a}}$ transcriptional repressors Bmi-1 and Id1 (Jacobs et al. 1999a; Ohtani et al. 2001) and increasing the activity of the $p16^{\text{INK4a}}$ transcription enhancers, Ets-2 and MITF (Fig. 11.3) (Loercher et al. 2005; Ohtani et al. 2001). Importantly, Bmi-1 is a direct transcription target of c-MYC, and reduced c-MYC signalling is associated with oncogenic N-RAS and B-RAF activity in melanocytes (Zhuang et al. 2008). Thus, decreased c-MYC activity leads to senescence by regulating Bmi-1 and $p16^{\text{INK4a}}$ (Guney et al. 2006; Jacobs et al. 1999b). Further, Bmi-1 deficiency inhibits stem cell renewal via $p16^{\text{INK4a}}$ induction in mice and Bmi-1 downregulation was associated with increased $p16^{\text{INK4a}}$ expression in human keratinocytes derived from older individuals (Cordisco et al. 2010; Molofsky et al. 2003, 2005; Park et al. 2003).

11.4.3

The Senescence-Associated Secretory Network

Senescent cells secrete a complex range of chemokines, interleukins, proteases and growth factors that are associated with inflammation and malignancy. Collectively, these senescence-associated soluble factors have been termed the senescence-associated secretory phenotype (SASP) and the senescence-messaging secretome (SMS) (Coppe et al. 2008; Gorgoulis and Halazonetis 2010; Kuilman and Peeper 2009). Detailed lists of SASP factors have been provided in several recent articles and are briefly summarised in Table 11.1 (Coppe et al. 2010; Gorgoulis and Halazonetis 2010; Kuilman and Peeper 2009). This review will focus on the role of SASP in senescence and its relationship with DNA damage response, p53 and pRb pathways.

SASP is not triggered by senescence, but is initiated in response to persistent DNA damage signalling. SASP requires upstream elements of the DNA damage response cascade, including NBS1 (a sensor of DNA damage), ATM kinase and its target Chk2, but not

Table 11.1 The senescence-associated secretory phenotype. A subset of factors significantly altered during senescence

<i>Soluble signalling factors</i>	
Interleukins	IL-6, -7, -1a, -1b, -13, -15
Chemokines	IL-8; GROa, -β; MCP-2, -4; HCC-4; cotaxin-3; MIP-3a, -1a; IGFBP-2, -3, -4, -5, -6, -7; G-CSF; GM-CSF
Growth factors	EGF; bFGF; HGF; IGF, SCF; TGFβ, NGF; VEGF
Non-protein factors	Nitric oxide, reactive oxygen species
<i>Serine proteases</i>	
	MMP-1, -3, -10, -12, -13, -14; TIMP-1, -2; PAI-1, -2
<i>Secreted insoluble factors</i>	
	Fibronectin, collagen, laminin

the downstream DNA damage signalling target p53. Thus, cells induced to senesce in response to p16^{INK4a} in the absence of DNA damage, do not initiate SASP. Similarly, oncogenic RAS promotes senescence with limited SASP in ATM-deficient cells (Rodier et al. 2009). Although SASP is not restricted to the senescence programme, it can reinforce senescence growth arrest. For instance, human fibroblasts depleted for IL-6 bypassed B-RAF^{V600E}-induced senescence and showed a strong suppression of other inflammatory regulators including IL-8, IL-1α and IL-1β (Kuilman et al. 2008). SASP molecules IL-8 and the chemokine receptor 2 (CXCR2) ligands support senescence by boosting the DNA damage response, while IGFBP7, IL-6 and PAI-1 contribute to senescence by inhibiting proliferative and mitogenic pathways (Acosta and Gil 2009; Acosta et al. 2008); Kortlever et al. 2006; Kuilman et al. 2008; Wajapeyee et al. 2008). Thus, the onset of a protracted DNA damage response controls senescence by initiating a rapid p53- and/or pRb-dependent proliferative arrest, followed by induction of a DNA damage responsive cytokine secretory response (Fig. 11.2).

The secretion of soluble factors into the extracellular environment can also have protumourigenic effects; this probably reflects a complex combination of cellular and genetic context along with the level of SASP activity. SASP factors such as IL-6 and IL-8 can promote cancer progression by stimulating proliferation, angiogenesis, invasiveness and inducing epithelial-mesenchymal transition (Ancrile et al. 2007; Coppe et al. 2008; Kuilman et al. 2008; Rodier et al. 2009). Others, such as matrix metalloproteinases promote breast tumourigenesis by altering the differentiation of epithelial tumour cells and increasing tumour cell migration by weakening tissue integrity (Acosta and Gil 2009; Liu and Hornsby 2007). The combined loss of p53 with the aberrant activation of RAS amplifies and accelerates the development of SASP and this coincides with the potent stimulation of growth, invasiveness and epithelial-mesenchymal transition of non-aggressive human cancer cells (Coppe et al. 2008). *In vivo* cytokine secretion is a feature of preneoplastic lesions of the colon and breast, which display markers of DNA damage and senescence, while IL-6 expression correlates with ATM (DNA damage sensor) kinase activity in invasive ductal breast carcinomas (Kuilman et al. 2008; Rodier et al. 2009).

Finally, the innate immune system can be activated by SASP inflammatory cytokines and can effectively restrict oncogenic SASP activity by clearing damaged and senescent cells (Xue et al. 2007). The accumulation of inflammatory cytokines is associated with several age-associated diseases, and it is not surprising that accumulation of SASP factors such as IL-6, TGF β , PAI and fibronectin also correlate with ageing (Carrieri et al. 2004; Ershler and Keller 2000; Goldstein et al. 1994; Herbig et al. 2006; Rasoamanantena et al. 1994; Rossi et al. 2007). Although the precise impact of the SASP *in vivo* remains unresolved, these data indicate that damaged senescent cells mount a complex cytokine response that communicates with neighbouring cells, modifies the tissue microenvironment and leads to multiple pathologies. In particular, cytokines may reinforce senescence in surrounding non-malignant cells, generate a potent immune response to clear damaged cells, affect the function of stem cells or even promote tumourigenesis of high-grade pre-malignant and malignant cells in a paracrine manner (Fig. 11.2).

11.4.4

p53- and p16^{INK4a}-Independent Oncogene-Induced Senescence in Melanocytes

Recent reports have shown that pro-oncogenic forms of the H- and N-RAS GTPases, and their downstream kinase B-RAF, are not functionally equivalent and each may induce senescence via distinct programmes that do not require pRb or p53. Certainly, the inactivation of p16^{INK4a}, pRb or p53 did not prevent cell cycle arrest, DNA damage signalling or SA- β -gal activity in human melanocytes transduced to express these oncogenes (Denoyelle et al. 2006; Haferkamp et al. 2009a, b; Michaloglou et al. 2005; Zhuang et al. 2008). One alternative pathway appears to involve the endoplasmic reticulum-associated unfolded protein response (UPR). Ectopic expression of H-RAS^{G12V} in human melanocytes induced the UPR, and inhibition of this response (by silencing expression of several UPR proteins) suppressed H-RAS-induced senescence (Denoyelle et al. 2006). The RAS-mediated activation of the PI3K pathway was required for UPR induction and this was most potently achieved by oncogenic H-RAS, as UPR was poorly induced by N-RAS and not activated by B-RAF. Consistent with these data, Spitz naevi, expressing mutated H-RAS, showed a significantly greater expansion of the endoplasmic reticulum and activation of the UPR compared to benign naevi expressing mutated B-RAF or N-RAS (Denoyelle et al. 2006). It has been suggested that UPR-induced senescence may account for the bias against H-RAS mutations in melanoma (Denoyelle et al. 2006), although it should be noted that the efficiency of UPR signalling declines during ageing (Naidoo 2009a, b), and melanomas seem to have adapted to the presence of extreme endoplasmic reticulum stress (Hersey and Zhang 2008; Jiang et al. 2007).

Another alternative senescence pathway has been proposed for oncogenic B-RAF in human melanocytes. Ectopic expression of the oncogenic transcription factor c-MYC was found to partially rescue B-RAF^{V600E}-induced senescence, and more weakly, N-RAS^{Q61R}-induced senescence of human melanocytes. c-MYC did not influence the UPR pathway and this may account for the inability of c-MYC to overcome N-RAS-induced senescence (Zhuang et al. 2008). Suppression of c-MYC has been shown to induce senescence in several mouse tumour models, including lymphoma, osteosarcoma and hepatocellular carcinoma (Wu et al. 2007), and in human melanoma cells expressing B-RAF^{V600E} or

N-RAS^{Q61R} (Zhuang et al. 2008). The requirement for p16^{INK4a} or p53 in senescence caused by c-MYC depletion remains controversial; intact p16^{INK4a}, pRb and p53 were required in mouse tumours for the initiation of senescence by c-MYC inactivation (Wu et al. 2007), whereas c-MYC depletion led to senescence of p16^{INK4a}-null and p53-null human melanoma cells (Zhuang et al. 2008). The impact of p16^{INK4a} and p53 expression on c-MYC activity in human melanocytes remains to be investigated. Importantly, c-MYC is frequently upregulated in human tumours and is up to sevenfold higher in metastatic melanomas compared to benign naevi (Zhuang et al. 2008).

The anti-senescence function of c-MYC requires phosphorylation of MYC at Ser-62 by cyclin E/CDK2. CDK2 acts as a c-MYC transcription cofactor altering the MYC-dependent regulation of genes, such as Bmi-1, p16^{INK4a} and p21^{Waf1}, all of which participate in senescence control. Inhibition of CDK2 leads to the upregulation of p16^{INK4a} and p21^{Waf1} and the repression of Bmi-1. Thus MYC can induce senescence in MEFs lacking CDK2, but not in wild type MEFs (Hydbring et al. 2010; Hydbring and Larsson 2010). This has important implications, as the inhibition of CDK2 (via pharmacological inhibition or induction of the CDK2 inhibitor p27^{Kip1}) can drive MYC-transformed cells into senescence and prevents c-MYC from bypassing RAS-induced senescence (reviewed in Hydbring and Larsson 2010). Accordingly, depletion of CDK2 delayed Myc-induced B-cell lymphomas, neuroblastoma cells, breast cancers and melanoma cell lines (Campaner et al. 2010; Deans et al. 2006; Du et al. 2004; Molenaar et al. 2009) and deletion of Skp2 (an oncogenic E3 ligase that targets p27^{Kip1} and MYC for degradation) suppressed tumourigenesis through senescence (Lin et al. 2010).

11.5

Re-establishing Senescence Mechanisms

The concept of re-instating senescence as a cancer therapy is being approached with caution because the retention of damaged senescent cancer cells that are metabolically active and capable of secreting cytokines and growth factors could be harmful. It is important to consider, however, that many current cancer treatments cause senescence as a result of acute DNA damage (Roberson et al. 2005; te Poele et al. 2002), and the presence of senescent cells in colon carcinomas contributed to improved overall outcome to therapy (Haugstetter et al. 2010). The existence of sporadic senescent cells in tumours may indicate a retained susceptibility to senescence induction and this may translate to sensitivity to senescence-inducing therapies.

Senescence can be triggered by reactivating tumour suppressor molecules or targeting oncogenes. For instance, reactivation of p53 induces a potent senescence response in sarcomas and liver carcinomas that were subsequently cleared by the innate immune system (Ventura et al. 2007; Xue et al. 2007). Presumably, SASP molecules were responsible for the activation of the immune cells. Targeting the oncogenic E3-ubiquitin ligase, Skp2, triggered senescence in H-RAS^{G12V} expressing or PTEN-deficient prostate cancer cells (Lin et al. 2010). Likewise, deleting CDK2 induced senescence in c-MYC expressing MEFs (Campaner et al. 2010). Importantly, senescence was also triggered in these oncogenic

model systems with small molecule inhibitors of Skp2 and CDK2 function (Lin et al. 2010). Moreover, the pharmacological induction of senescence has been shown to suppress the *in vivo* growth of prostate cancer cells (Alimonti et al. 2010). Mouse models of chemotherapy have also shown that MYC-initiated lymphomas respond to cyclohexamide by inducing tumour cell senescence, which contributed to better prognosis (Schmitt et al. 2002). This suggests that delivery of chemotherapy to cells undergoing senescence could be used to augment senescence or to transform cell cycle arrest into cell death.

11.6 Conclusions

Together, these data confirm that senescence plays an important role in suppressing tumorigenesis and possibly predicting treatment outcome. The long-term impact of senescent tumour cells *in vivo* remains largely unknown, and much more work is needed to resolve the complex regulation of the pro- and anti-tumorigenic activities of senescent cells. The finding that the senescent phenotype can be uncoupled from cell cycle arrest suggests that it may be possible to minimise the negative aspects of senescence (Dulic et al. 2000; Rodier et al. 2009; Wang et al. 2004). For instance, p53 activity and the miR-146 class of microRNAs suppress excessive SASP activity and may contribute to its pro-senescence functions (Bhaumik et al. 2009; Coppe et al. 2008). Certainly, the therapeutic potential of senescence will most likely depend on establishing a new equilibrium that promotes tumour suppression by cell cycle arrest and cell death.

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Abstract The hypothesis that tumor initiation and growth are driven by a subpopulation of malignant cells, that is, cancer stem cells (CSCs), has received considerable attention. The CSC concept predicts that the design of novel therapies that ablate CSCs or target CSC-specific protumorigenic signaling pathways might result in more durable therapeutic responses in cancer patients than those achieved by therapeutic approaches targeted predominantly at non-CSC tumor bulk populations. Evidence for the existence of CSCs has been generated in a broad range of human malignancies, including in human melanomas as malignant melanoma-initiating cells (MMICs). Several recent studies have documented a specific relationship of MMICs to melanoma progression and therapeutic resistance. Moreover, proof-of-principle for the potential therapeutic utility of targeting MMICs has been provided by demonstrating that selective killing of MMICs can inhibit tumor growth. The biological mechanisms by which MMICs may fuel the tumorigenic process have recently started to be elucidated. In this chapter, we will discuss the potential importance of these translationally relevant research developments for the identification of novel therapeutic targets and prognostic biomarkers in human malignant melanoma.

12.1 Introduction

The hypothesis that cancers and tissue stem cells might share several biological traits is many decades old (Bruce and Van Der Gaag 1963; Hamburger and Salmon 1977; Park et al. 1971). Indeed, the defining stem cell traits of (a) self-renewal, (b) differentiation, and (c) potential to proliferate nearly indefinitely are commonly observed among cancer subpopulations (Reya et al. 2001). Moreover, interactions with stromal elements and signaling

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pathways that govern physiologic stem cell behavior were also found to play important roles during tumor development (Frank et al. 2010). Together, these observations have led to the cancer stem cells (CSC) theory of tumor initiation and growth, which postulates that the tumorigenic process relies on a reservoir of self-renewing, aggressive cells that confer clinical virulence, that is, CSCs (Reya et al. 2001). Such tumorigenic minority populations or CSCs have since been characterized in a growing number of tumor entities (Schatton et al. 2009), including in human melanoma (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Schatton et al. 2008).

In this chapter, we will review the current knowledge of CSCs and their biological features with particular emphasis on human malignant melanoma, and discuss controversial aspects of the CSC theory. Furthermore, we will explore the implications of the findings of melanoma as a CSC-driven disease for the development of more effective treatment modalities for melanoma patients.

12.2

What Is a “Cancer Stem Cell”?

It has long been established that tumors comprise multiple phenotypically and functionally distinct populations of cancer cells (Hanahan and Weinberg 2000). Several theories have been put forward to account for the occurrence of such tumor heterogeneity. According to the classic view of tumorigenesis, tumor heterogeneity can be explained by both intrinsic factors (i.e., progressive accumulations of genetic alterations over time, see also Chap. 4) and extrinsic stimuli (e.g., distinct cues from the tumor microenvironment, see also Chap. 14, Hanahan and Weinberg 2000). The so-called stochastic theory of tumor initiation postulates that all malignant cells within a cancer, regardless of their phenotype, possess equivalent capacities to proliferate, form new tumors, and cause relapse (Nowell 1976). The CSC hypothesis, on the other hand, provides an alternative explanation for tumor heterogeneity (Reya et al. 2001): It posits that cancers, like physiologic tissues, are organized as developmentally defined hierarchies of cells with divergent differentiation features and disparate capabilities for self-renewal and neoplastic proliferation. The CSC concept thus proposes that only a subpopulation of tumor cells within a cancer, namely CSCs, bears the competence to fuel tumor growth by continuously undergoing self-renewal and differentiation, whereas the bulk of differentiated cancer components lacks the capacity for tumor initiation and unlimited proliferation (Schatton et al. 2009) (Fig. 12.1).

According to a consensus definition (Clarke et al. 2006), a CSC is a cell within a tumor that possesses the capacity to undergo both self-renewing cell divisions that expand the CSC pool, and cell divisions that result in more differentiated cancer cell progeny. Therefore, CSCs can only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor (Clarke et al. 2006). Accordingly, the experimental characterization of a putative CSC population relies on the use of an *in vivo* model system that allows for a rigorous confirmation of the traits used to define CSCs. The gold standard assay that fulfills this criterion is serial xenotransplantation at limiting dilution of marker-defined clinical cancer subpopulations into an orthotopic site

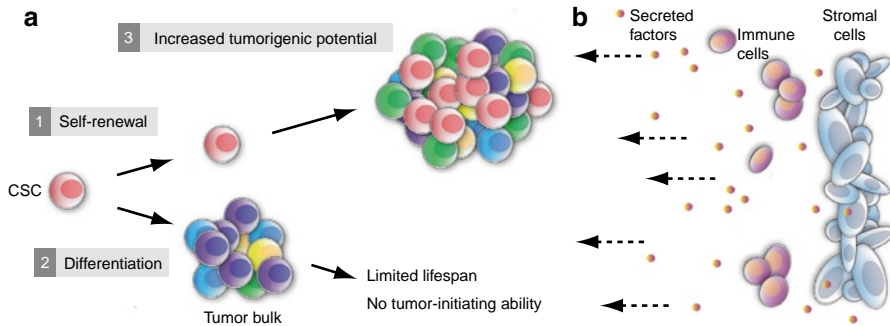


Fig. 12.1 *The cardinal features of CSCs.* Illustrated are (a) the defining features of CSCs, that is, (1) self-renewal capacity, (2) differentiation capacity, and (3) increased tumorigenic potential. (b) TME characteristics, that is, secreted factors, host immunity, and stromal cell interactions also regulate CSC-dependent tumor growth

of immunocompromised mice (typically NOD/SCID), which although imperfect is considered the best experimental system to evaluate CSC activity (Clarke et al. 2006). Using this approach, CSCs capable of sustained self-renewal and tumor propagation were first described in cancers of the hematopoietic lineage (Bonnet and Dick 1997). These initial studies demonstrated that it is possible to isolate from a single tumor sample two distinct cancer subpopulations that differ in their cell surface antigen profile and their tumor-seeding properties: (1) a CSC-enriched subset, as defined by its exclusive ability to self-renew as well as differentiate into nontumorigenic cancer cell progeny and its competence to seed new tumors upon serial xenotransplantation, and (2) the bulk of tumor cells that lack the capacity to generate tumors in animal hosts (Bonnet and Dick 1997). Subsequent studies extended these findings to a variety of additional hematological malignancies and solid tumor entities (Al-Hajj et al. 2003; Castor et al. 2005; Chan et al. 2009; Cox et al. 2004, 2007; Dalerba et al. 2007; Hermann et al. 2007; Ishikawa et al. 2007; Li et al. 2007; O'Brien et al. 2007; Prince et al. 2007; Ricci-Vitiani et al. 2007; Singh et al. 2004; Suva et al. 2009; Yang et al. 2008; Zhang et al. 2008), including malignant melanoma (Boiko et al. 2010; Schatton et al. 2008). Hierarchical tumor organization was recently also confirmed in syngeneic mouse models in which only fractions of murine tumor cells possessed the fundamental CSC features of extensive self-renewal, differentiation, and enhanced tumorigenic capacity (Cho et al. 2008; Deshpande et al. 2006; Held et al. 2010; Wu et al. 2008).

Despite these advances in our understanding of functional tumor heterogeneity, the CSC model remains a topic of considerable controversy (Hill 2006; Jordan 2009). Some of this controversy appears to arise from uncertainty regarding the term “CSC.” For instance, the term CSC has been interpreted to mean that the cellular precursors of such tumorigenic subpopulations were originally physiologic stem cells, which accumulated genetic alterations resulting in cancerous transformation. While this may be the case in some malignancies (Barker et al. 2009; Zhu et al. 2009), CSCs in other cancers may originate from more differentiated cells that reacquired stem-like properties through a series of mutagenic events (Huntly et al. 2004; Jamieson et al. 2004; Krivtsov et al. 2006). Additionally,

differentiation in the context of CSC biology does not refer to multipotent differentiation plasticity as it occurs during organogenesis or physiologic tissue regeneration, but rather to the ability of CSCs to give rise to cancer cells that lack tumor-initiating capacity (Clarke et al. 2006). Furthermore, in contrast to physiologic stem cells, which represent only a small cellular fraction of a particular tissue, CSCs may represent larger relative proportions of a total cancer cell population, depending on tumor type, variance of genetic alterations, and stage of disease progression (Gupta et al. 2009). In support of this notion, the frequency of leukemic CSCs varied more than 100-fold between distinct patient specimens (Bonnet and Dick 1997). CSC representation may also vary within a single cancer specimen, in which undifferentiated regions may harbor larger numbers of CSCs compared to more differentiated tumor areas (Gupta et al. 2009). Importantly, the number of cells needed to initiate a tumor is not part of the CSC definition (Reya et al. 2001; Schatton et al. 2009). Hence, a larger relative proportion of tumorigenic cells does not contradict the CSC model of tumor initiation and growth. Given the potential confusion associated with the term “CSC,” many investigators in the field refer to them as tumor-initiating or tumor-propagating cells (Clarke et al. 2006).

The determination of relative CSC frequencies is influenced by the experimental model system used to assess cancer “stemness” (Boiko et al. 2010; Bonnet and Dick 1997; Lapidot et al. 1994; O’Brien et al. 2007; Quintana et al. 2008, 2010; Ricci-Vitiani et al. 2007; Schatton et al. 2008; Shmelkov et al. 2008). In this regard, it has been established for some time that biological aspects of the tumor microenvironment, including growth factor availability, extracellular matrix (ECM) composition, or the degree of vascularization, as well as host immunocompetence can control the tumorigenic potential of cancer subpopulations (Scadden 2006) (Fig. 12.1). Given this dependence of a defining CSC feature on microenvironmental factors and the immune status of the host, it is not surprising that animal models that offer a more hospitable microenvironment for tumor growth – that is, through the exogenous addition of ECM factors (e.g., Matrigel) (Quintana et al. 2008) and/or the use of more severely immunocompromised mice (Bonnet and Dick 1997; Quintana et al. 2008) – can yield higher relative CSC counts compared to CSC frequencies assessed in the absence of cografted stromal factors in more immunocompetent mouse models (Lapidot et al. 1994; Quintana et al. 2008; Schatton et al. 2008). Based on these considerations, more permissive xenotransplantation conditions might be inadequate for the accurate assessment of CSC biology as it occurs in humans, as they could enable non-CSCs to initiate and maintain experimental tumor growth (Gupta et al. 2009).

The tumor biospecimens used for the isolation and characterization of putative CSC populations also influence the assessment of CSC frequency (Gupta et al. 2009). For instance, utilizing tumor cell isolates from tumor xenografts that have been passaged *in vivo* for extended periods of time (Quintana et al. 2008, 2010) in lieu of fresh patient-derived tumor samples likely obscures the accurate assessment of CSC frequencies and biological functions (Boiko et al. 2010). Indeed, Boiko and colleagues demonstrated that both *in vitro* and *in vivo* passaging of melanoma cells can result in the emergence of tumorigenic subclones that drive experimental tumor growth independent of their immunophenotype (Boiko et al. 2010). Similarly, tumor cell lines may have lost the hierarchical structure of the primary tumor from which they originated (Zhou et al. 2009). Variations in tumor dissociation, isolation, and/or inoculation techniques could also account for

differences in calculated CSC frequencies between different laboratories (Shackleton 2010). Moreover, the use of tumor specimens from patients with more advanced disease (Quintana et al. 2008), which have been demonstrated to contain elevated CSC numbers compared to primary tumors (Schatton et al. 2008), could yield higher estimated CSC frequencies (Boiko et al. 2010).

Clearly, a considerable variability has been observed in the course of CSC identification efforts with regard to estimated frequencies of tumorigenic cells depending on the mouse model used. While the relative frequency of CSCs is not directly relevant for their identification as it is not a CSC-defining feature, it is nevertheless important to define the factors responsible for this variability, because such studies could help identify additional CSC-specific functions (Schatton et al. 2009). For example, the tumor microenvironment, including that of an immunocompromised host, may not only govern the tumorigenic potential of cancer subpopulations, but also additional CSC-defining traits, including differentiation and self-renewal (Postovit et al. 2006). It is thus possible that a very permissive milieu for tumor growth may not only enable non-CSCs to seed cancers, but could also facilitate the “de-differentiation” of such nontumorigenic bulk populations into CSC-phenotype expressing cells (Hoek and Goding 2010). The conversion of non-CSCs into CSC-like cells may, however, not occur in more hostile cancer environments observed in patient tumors, which are typically characterized by low nutrient availability, marked levels of hypoxia, necrosis, and inflammation, and the presence of antitumor immune responses. Only a minority of tumor cells, that is, CSCs, might possess the ability to survive and fuel the tumorigenic process under these selective pressures. In support of this hypothesis, hypoxia-inducible factors (HIFs) are preferentially expressed in CSCs, and blockade of HIF2 α on glioma CSCs attenuated their potential to initiate experimental tumors (Li et al. 2009b). Furthermore, viable glioma CSCs preferentially localize to perivascular niches in both clinical and experimental cancer specimens (Calabrese et al. 2007). The bulk of tumor cells within cancerous lesions that have reached a significant size, on the other hand, is often necrotic or undergoes apoptosis (Cotter 2009), indicating that their conversion into apoptosis-resistant stem-like cancer cells does not commonly occur under these conditions.

The complexity of CSC biology and its implications for the type of animal model utilized for their characterization is further highlighted by the identification of novel CSC functions (Frank et al. 2010), including their intrinsic property to evade or actively modulate antitumor immune responses (Chan et al. 2009; Di Tomaso et al. 2010; Majeti et al. 2009; Schatton and Frank 2009; Schatton et al. 2010; Todaro et al. 2009; Wei et al. 2010a, b). Such mechanisms, which may confer selective growth advantages to the CSC pool, need to be taken into consideration when designing biologically relevant assays for their characterization. These findings highlight the importance of establishing translationally relevant assays for the study of CSCs, which accurately mimic the environmental factors found in clinical cancers (e.g., presence of human stromal cells, low-nutrient levels, necrosis, hypoxia, and relatively intact antitumor immunity) rather than further deviating from the naturally occurring microenvironmental characteristics. Current xenotransplantation protocols and animal models for the study of tumorigenic subpopulations that do not accurately reflect the pathological environment of spontaneously occurring human malignancies might favor niche-independent tumor growth (Quintana et al. 2008). Similarly, the

so-called tumor sphere culture assay, which favors anchorage-independent *in vitro* passaging of cancer cells, and which has been proposed to serve as a surrogate tool for the identification of tumor-initiating cells (Fang et al. 2005; Keshet et al. 2008; Perego et al. 2010), represents an inadequate stand-alone assay for the relevant study of CSC biology in the absence of an *in vivo* confirmation of CSC-defining traits of a marker-defined tumor subset (Schatton and Frank 2010).

Although much work is still required to fully characterize CSCs and to reach consensus about clinically relevant model systems for their study, efforts directed toward identifying strategies that effectively target tumorigenic minority populations could significantly enhance current treatment modalities. In the following sections, we will discuss how the study of MMICs can yield new insights into melanomagenesis that could potentially lead to the development of improved melanoma treatment strategies.

12.3

Melanoma Stem Cells: Growing Evidence for Their Clinical Relevance

The study of CSCs in human malignant melanoma has recently elicited considerable interest. Given its marked heterogeneity, pronounced resistance to conventional anticancer therapy, and highly aggressive behavior, it has been speculated for some time that melanoma, like numerous additional solid cancers, may follow the CSC model of tumor initiation and growth (Hendrix et al. 2003a; Schatton and Frank 2008). Although the survival rate of patients diagnosed with melanoma has improved over the past decades (Linos et al. 2009), melanoma remains the most lethal form of skin cancer and melanoma incidence is increasing faster than any other cancer worldwide (Jemal et al. 2010) (see also Chaps. 16 and 17). Taken together with the minimal therapeutic benefit of current treatment strategies for patients with advanced stage disease (Chin et al. 2006), these facts clearly indicate the urgency for novel strategies to eradicate the melanoma subpopulation in which clinical virulence and therapy resistance reside, which may coincide with MMICs.

Initial support for the potential existence of MMICs came from the demonstration that CSC phenotype-expressing cancer subpopulations are present in melanoma cell lines and clinical melanomas (Frank et al. 2005). Specifically, this study showed that a number of stem cell markers, including the prospective CSC determinant, **CD133** (Hermann et al. 2007; O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Singh et al. 2004; Suva et al. 2009), were overexpressed by chemoresistant tumor subsets among heterogeneous malignant melanoma cultures and within clinical melanoma biospecimens (Frank et al. 2005). Subsequently, Monzani and colleagues showed that CD133⁺ melanoma fractions had the preferential ability to initiate primary tumor formation in NOD/SCID mice compared to CD133⁻ melanoma bulk populations (Monzani et al. 2007), demonstrating the existence of tumorigenic melanoma subpopulations. The authors of the study did not examine, however, whether CD133⁺ melanoma subsets were capable of self-renewal and differentiation in serial xenotransplantation experiments (Monzani et al. 2007). In the absence of stringent *in vivo* assays aimed at dissecting these key CSC defining features, no definitive conclusions can be made about the potential relationship of CD133⁺ or other melanoma minority populations to MMICs. This limitation

also applies to a study characterizing melanoma subpopulations based on their ability to grow in spheroid bodies *in vitro* (Fang et al. 2005). Melanoma cells isolated from such “melano-spheres” preferentially expressed the marker of mature B cells, **CD20**, and were more tumorigenic when grafted to immunodeficient mice compared to melanoma cells grown as adherent *in vitro* cultures (Fang et al. 2005). While the results by Fang and colleagues (Fang et al. 2005) did not establish the existence of MMICs, they suggested, however, that melanomas might comprise functionally distinct subpopulations with divergent tumorigenic capacities.

Unequivocal evidence for the existence of melanoma CSCs, capable of long-term self-renewal and differentiation, was first provided when the chemoresistance mediator (Cheung et al. 2011; Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Huang et al. 2004; Yang et al. 2010) and cell surface molecule, **ABCB5**, was established as a prospective biomarker of MMICs (Schatton et al. 2008). This study demonstrated that **ABCB5⁺** melanoma fractions, but not **ABCB5⁻** melanoma bulk populations, isolated from patient biopsies were capable of initiating primary and secondary neoplasms upon serial xenotransplantation at limiting dilution into NOD/SCID mice (Schatton et al. 2008). In addition, **ABCB5⁺** melanoma cells were capable of generating phenocopies of the original patient tumors, indicating their ability to both self-renew (i.e., to maintain the **ABCB5⁺** cell pool by generating more copies of themselves) and differentiate (i.e., to give rise to more differentiated, **ABCB5⁻** tumor cell progeny, incapable of initiating experimental tumor growth) upon serial xenotransplantation (Schatton et al. 2008). These results thus established that **ABCB5⁺** melanoma subpopulations represent MMICs (Zabierowski and Herlyn 2008) as per the CSC consensus definition (Clarke et al. 2006) outlined above.

To further confirm the selective capacity of **ABCB5⁺** MMICs for enhanced tumorigenic growth, self-renewal, and differentiation, genetic lineage tracking was performed in human melanoma to NOD/SCID mouse xenotransplantation experiments employing genetically encoded DsRed (red fluorescent protein) and EYFP (enhanced yellow–green fluorescent protein) labeling of MMICs and melanoma bulk populations, respectively (Schatton et al. 2008). Xenotransplantation of **ABCB5⁺DsRed⁺** melanoma cells and **ABCB5⁻EYFP⁺** tumor bulk components, reconstituted at naturally occurring ratios, resulted in markedly increased relative frequencies of DsRed⁺ cells of **ABCB5⁺** origin in melanoma xenografts (Schatton et al. 2008), confirming the enhanced tumorigenic capacity of **ABCB5⁺** MMICs. In addition, these lineage-tracing experiments revealed a tumor hierarchy, in which **ABCB5⁺** cells had the ability to generate both **ABCB5⁺** and **ABCB5⁻** tumor progeny, whereas **ABCB5⁻** melanoma cells exclusively gave rise to more copies of themselves (Schatton et al. 2008). This work thus demonstrated that two fundamentally different cell types with divergent tumorigenic potentials existed within melanomas, only one of which was capable of self-renewal and differentiation – that is, **ABCB5⁺** MMICs. To further establish that the melanoma subpopulation marked by **ABCB5** is required for efficient tumor growth, as would be anticipated if the MMIC model were valid, we examined whether selective ablation of MMICs could inhibit tumor development (Schatton et al. 2008). Indeed, administration of an anti-**ABCB5** monoclonal antibody into nude mice xenografted with human melanomas impaired tumor initiation and slowed tumorigenic growth via antibody-dependent cell-mediated cytotoxicity (ADCC) directed at **ABCB5⁺** MMICs (Schatton et al. 2008). These results provided proof-of-concept for the potential therapeutic utility of targeting MMICs and provided evidence that melanoma is a CSC-driven disease.

In the same study, using an established melanocytic tumor tissue microarray (Kim et al. 2006), a positive correlation between ABCB5⁺ melanoma cell frequency and clinical melanoma progression was established (Schatton et al. 2008). Specifically, there was higher ABCB5 expression in primary melanomas versus benign nevi, and in metastatic melanomas versus primary melanomas, showing that ABCB5 correlates with neoplastic progression (Schatton et al. 2008). A role for ABCB5 in clinical melanoma initiation/progression has recently also been established by additional laboratories (Gazzaniga et al. 2010; Sharma et al. 2010), and these findings have been extended to acral melanomas (Vasquez-Moctezuma et al. 2010) and *in vivo* human melanoma model systems (Fukunaga-Kalabis et al. 2010; Ma et al. 2010). ABCB5 expression also correlates with neoplastic progression in additional malignancies, including hepatocellular carcinoma, where it serves as a major independent clinical biomarker of poor survival (Cheung et al. 2011), warranting similar studies in human melanoma. The potential importance of ABCB5 as a biomarker of metastatic melanoma progression and disseminated disease is also supported by an additional recent study from our laboratory (Ma et al. 2010): ABCB5 mRNA could be detected in peripheral blood mononuclear cell preparations from human stage IV melanoma patients but not healthy controls (Ma et al. 2010). Similarly, human ABCB5 mRNA expression could also be detected among peripheral blood mononuclear cells isolated from NOD *scid* gamma (NSG) recipients of subcutaneous human melanoma xenografts (Ma et al. 2010), indicating that circulating melanoma-initiating cells might be present in both murine models and stage IV melanoma patients. Indeed, viable circulating tumor cells (CTCs), isolated from the blood circulation of tumor-bearing hosts, were capable of primary melanoma and metastasis formation in serial xenotransplantation experiments (Ma et al. 2010). Strikingly, CTCs capable of melanoma initiation demonstrated significantly increased ABCB5 expression levels compared to primary tumor xenografts and metastatic lesions (Ma et al. 2010). These results provided initial evidence that circulating melanoma cells are tumorigenic and capable of metastasis formation, and showed that CTCs are enriched for MMICs. Together, these results highlight that ABCB5 identifies clinically important disseminated melanoma subpopulations that warrant future research investigations regarding their potential role as a novel diagnostic and therapeutic biomarker (Ma et al. 2010).

Additional support for the CSC properties of ABCB5⁺ melanoma fractions has also come from syngeneic melanoma mouse models (Ehira et al. 2010). Specifically, a tumorigenic B16 murine melanoma cell line variant demonstrated increased *Abcb5* expression upon *in vivo* tumor formation (Ehira et al. 2010) alongside with other markers previously shown to enrich for murine MMIC-like cells with increased clonogenic and tumorigenic potentials (Dou et al. 2007). Taken together, these findings indicate that murine *Abcb5*⁺ B16 melanoma subpopulations, like human ABCB5⁺ MMICs, represent CSC-like cells that amplify during the tumorigenic process (Ehira et al. 2010). Similarly, the ABCB5 gene was also preferentially expressed by human melanomas with high *in vivo* tumorigenic capacity in human to murine xenotransplantation experiments (Hoek et al. 2004, 2008). Consistent with these findings, human melanoma subpopulations with enhanced *in vitro* clonogenic and self-renewal capacities demonstrated increased ABCB5 expression levels (Keshet et al. 2008). In a separate study, induction of terminal differentiation of human melanoma cells resulted in a significant decrease in ABCB5 expression

concomitant with reduced proliferation and enhanced susceptibility to chemotherapeutic agents (Botelho et al. 2010). Thus, both *in vitro* and *in vivo* substantiation of the CSC properties of ABCB5⁺ melanoma fractions was established in settings that are independent of the foreign host milieu given in human to mouse xenotransplantation assays (Botelho et al. 2010; Ehira et al. 2010; Keshet et al. 2008).

Recently, additional evidence that melanoma follows the CSC model of tumor initiation and growth was provided in serial human melanoma to mouse xenotransplantation experiments by two laboratories (Boiko et al. 2010; Boonyaratanakornkit et al. 2010). Boiko et al. demonstrated that MMICs can be prospectively isolated from melanoma patient specimens based on their expression of the nerve growth factor receptor (NGFR) **CD271** (also known as p75) (Boiko et al. 2010). Serial xenotransplantation of CD271⁺ melanoma subsets into engrafted human skin or bone in severely immunocompromised, T-, B-, and natural-killer deficient Rag2^{-/-}γ^{-/-} mice resulted in experimental tumor growth in 70% of injected animals (Boiko et al. 2010). In contrast, only 7% of mice inoculated with CD271⁻ melanoma cells developed tumors (Boiko et al. 2010). In addition, CD271⁺ melanoma subpopulations demonstrated both self-renewal and differentiation capacity through serial *in vivo* passaging (Boiko et al. 2010), consistent with the existence of a functional melanoma hierarchy (Dirks 2010). Importantly, CD271⁺ melanoma cells were also capable of metastasis *in vivo* (Boiko et al. 2010), suggesting a critical role for MMICs not only in tumor initiation but also in neoplastic progression. Remarkably, we found the MMIC markers ABCB5 (Schatten et al. 2008) and CD271 (Boiko et al. 2010) preferentially coexpressed on the same tumor subpopulation in human melanoma specimens (unpublished results). In addition, preferential coexpression of both markers has also been documented by the Weissman Laboratory at Stanford University (Alexander Boiko and Irving Weissman, personal communication), indicating significant overlap of these MMIC populations.

In a separate study, the detoxifying enzyme, aldehyde dehydrogenase (ALDH), was used to select for tumorigenic melanoma cell fractions (Boonyaratanakornkit et al. 2010). Of note, ALDH expression identifies CSCs in human breast cancer (Ginestier et al. 2007) and colon cancer (Carpentino et al. 2009; Huang et al. 2009). Similarly, high ALDH activity enriched for tumorigenic cells with sustained self-renewal capacity in human malignant melanoma was determined in both NOD/SCID and NSG recipients mice (Boonyaratanakornkit et al. 2010), confirming that not all melanoma cells are equally adept at tumor initiation, including in NSG recipients. Consistently, ALDH⁺ cells were found to give rise to ALDH⁻ melanoma progeny, while the conversion of ALDH⁻ into ALDH⁺ melanoma cells was rarely observed in a separate study (Prasmickaite et al. 2010), suggesting a preferential self-renewal ability of the ALDH^{high} cell pool. However, in this study ALDH activity did not enrich for melanoma subsets with enhanced tumorigenic potential (Prasmickaite et al. 2010), pointing to potential limitations of ALDH as a universal MMIC identifier. The relationship of ALDH expression to ABCB5 and/or CD271 is currently unknown and deserves further investigation, especially given the importance of the ALDH enzyme for conferring chemoresistance to alkylating agents (Vasiliou et al. 2004).

In summary, several recent studies independently demonstrated that not all melanoma cells possess equal capacities to initiate and maintain tumor growth in immunodeficient hosts (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Schatten et al. 2008). Rather, a distinct subpopulation of MMICs capable of self-renewal and differentiation exists (Boiko et al.

2010; Schatton et al. 2008). Both the ABCB5 (Schatton et al. 2008) and the CD271 (Boiko et al. 2010) surface molecules have been established as prospective biomarkers of MMICs, and important links of these unique melanoma subpopulations to neoplastic progression (Fukunaga-Kalabis et al. 2010; Gazzaniga et al. 2010; Ma et al. 2010; Schatton et al. 2008; Sharma et al. 2010; Vasquez-Moctezuma et al. 2010) and melanoma chemotherapy (Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Huang et al. 2004) and immunotherapy resistance (Boiko et al. 2010; Schatton et al. 2010) have emerged. Taken together, these findings highlight the clinical relevance of MMICs and emphasize the potential importance of research investigations directed at targeting these tumorigenic melanoma subpopulations to achieve better response rates in patients with advanced stage disease.

12.4

Melanoma Stem Cell Assays

Despite increasing knowledge regarding MMICs and their established relationship to melanocytic tumor progression and therapy resistance, a range of opinions exists with regard to the most relevant assay systems for CSC identification and frequency determination. MMIC frequencies in human melanoma to NOD/SCID mouse xenotransplantation experiments have been estimated to be approximately 1 in 10^6 cells among unfractionated melanoma populations when tumor formation was assessed 8 weeks post melanoma cell inoculation (Quintana et al. 2008; Schatton et al. 2008). Determination of experimental tumorigenicity in NOD/SCID recipients at 32 weeks post melanoma cell inoculation yielded a higher estimated frequency of tumorigenic melanoma cells of approximately 1 in 10^5 cells (Quintana et al. 2008). The use of more immunocompromised NSG hosts for assessment of tumor formation under otherwise equal experimental conditions yielded a further increase in estimated MMIC frequencies to approximately 1 in 5.5×10^3 cells among unsegregated patient-derived melanoma populations (Quintana et al. 2008). An even greater enrichment of tumorigenic cells was observed when melanoma cells were co-injected into NSG mice with matrigel (Quintana et al. 2008). Under these modified conditions, an average of 1 in 9 melanoma cells formed tumors at 8 weeks post melanoma cell inoculation (Quintana et al. 2008). While this study did not directly address CSC-defining features, such as sustained self-renewal and differentiation capacity in serial xenotransplantation experiments, the results support the view that alterations of the tumor environment can govern MMIC behavior.

Specifically, host environments characterized by absence of immune selective forces could permit tumor bulk populations, which do not normally initiate tumors and may not possess CSC-specific self-renewal and differentiation capacity to also cause experimental tumor growth. MMICs, on the other hand, might possess the preferential capacity to evade host immunosurveillance and initiate tumor growth (Schatton and Frank 2009), which would explain findings of lower estimated MMIC frequencies in more immunocompetent hosts. Indeed, several mechanisms by which MMICs evade antitumor immunity have recently been identified (Boiko et al. 2010; Schatton et al. 2010). For example, MMICs were found to express low to absent levels of immunogenic tumor-associated antigens (TAAs) (Boiko et al. 2010; Schatton et al. 2010), suggesting evasion from antitumor

immune responses directed at TAAs. Furthermore, ABCB5⁺ MMICs also preferentially blocked production of the proliferative cytokine IL-2 by cocultured lymphocytes (Schatton et al. 2010). Additional recently unraveled immunomodulatory functions of MMICs include the secretion of immunosuppressive factors and contact-dependent immunoregulatory mechanisms requiring engagement of immune-inhibitory surface molecules (Schatton et al. 2010), which will be discussed in more detail below. A relative immune privilege of CSCs was also demonstrated in alternative malignancies, including glioblastoma (Di Tomaso et al. 2010; Wei et al. 2010a, b), bladder cancer (Chan et al. 2009), colorectal carcinoma (Todaro et al. 2009), and leukemias (Majeti et al. 2009), indicating that immunomodulation might represent a common feature of CSCs (Schatton and Frank 2009). Clearly, the possibility of a CSC-driven tumor immune escape has profound implications not only for the development of improved cancer immunotherapeutic protocols but also for the design of biologically relevant assays for the study of CSC behavior. Specifically, assessment of tumor-initiating ability in the absence of antitumor immunity now appears to represent an inadequate assay system for the study of MMICs, because host immune environments incapable of immunologic tumor clearance might enable melanoma bulk populations to also initiate and sustain experimental tumor growth. Because preferential inhibition of IL-2 production is one mechanism by which MMICs evade antitumor immunity (Schatton et al. 2010), assessment of melanoma initiation in a murine model, that is, IL-2 receptor null (i.e., NSG mice) (Quintana et al. 2008) is not an appropriate environment for the accurate enumeration of MMIC frequency, because host immunity is abnormally impaired. In light of these considerations, it is not surprising that tumorigenicity experiments performed using more immunocompromised hosts with defective IL-2 receptors (i.e., NSG mice) (Quintana et al. 2008) yielded higher estimated MMIC frequencies compared to xenotransplantation assays utilizing more immunocompetent hosts (i.e., NOD/SCID mice) (Quintana et al. 2008; Schatton et al. 2008). In our view, tumor initiation in melanoma, one of the most immunogenic cancers, should be assessed in a setting that allows for host antitumor immune responses to occur. Ideally, model systems for the accurate assessment of MMIC biology might involve chimeric murine xenograft recipients that are orthotopically xenografted with human cancer cells into syngeneic human tissues of cancer origin in the presence of an adoptively transferred hematopoietic system originating from the same patient (Frank et al. 2010).

In addition to alterations of host antitumor immune response, co-injection of matrigel, a solution containing growth factors and ECM constituents (Kleinman and Martin 2005), accounted for a marked increase in tumorigenic capacity of unfractionated human melanoma cells and estimated MMIC frequencies in NSG mice (Quintana et al. 2008). In our view, these findings imply that the stage can be set for MMICs to appear more abundant by exposing them to microenvironmental stimulants such as ECM and growth factors. It is conceivable that in the absence of such growth-promoting signals, only MMICs might possess the ability to survive and as a consequence maintain the tumorigenic process. This possibility is indeed supported by findings in other CSC-driven cancers (Calabrese et al. 2007; Li et al. 2009b). The clinical cancer scenario is typically characterized by low nutrient and growth factor availability and deregulated ECM function (Hendrix et al. 2003b). It thus seems plausible that MMICs might preferentially produce both ECM and growth factors, thereby sustaining tumor maintenance. In support of this hypothesis, Duda and colleagues

demonstrated that circulating cancer cells, which are coated with autologous stromal components, including ECM factors, have the preferential capacity to seed distant metastasis and promote initial tumor growth compared to those cancer cells that are not incorporated in stromal elements (Duda et al. 2010). Upon establishment of a growth-promoting environment through ECM-expressing tumor subsets at the early stage of metastatic foci, additional cancer populations can also home to secondary tumor sites and contribute to neoplastic growth and progression during later disease stages (Duda et al. 2010). Similarly, genetic lineage tracing experiments revealed that melanoma bulk populations could furnish the growing tumor with cellular progeny in the presence of MMICs (Schatton et al. 2008). Thus, it is conceivable that the co-injection of growth-promoting factors to melanoma cell inocula may mask MMIC-specific functions and could allow most melanoma cells to initiate experimental tumor growth. Subpopulations of differentiated cancer cells that do not normally initiate tumors and do not display the ability for sustained self-renewal under clinical conditions may thus be able to do so in the presence of the appropriate microenvironment. Similarly, melanoma cells can give rise to induced pluripotent stem cells (iPSCs) through exogenous reprogramming factors (Utikal et al. 2009). While these findings clearly establish the *potential* of melanoma cells to generate iPSCs, it seems, however, unlikely that such gains of cellular plasticity occur naturally in human patients. In aggregate, these findings further highlight the importance of establishing translationally relevant assays for the study of MMICs, which accurately mimic the environmental factors found in clinical cancers (e.g., low-nutrient levels, necrosis, hypoxia, and relatively intact antitumor immunity) rather than further deviating from them. Assays that are very permissive to revealing the tumorigenic potential of melanoma cells might thus vastly overestimate MMIC frequencies. A major weakness of the hypothesis that the CSC model might not apply to melanoma (Quintana et al. 2008, 2010) is that MMICs must be rare. The observation that tumorigenic melanoma cells may be more common when assay conditions are modified does not imply that MMICs might not exist. Indeed, rarity is clearly not a defining criterion of CSCs according to the consensus definition (Clarke et al. 2006).

While the above outlined differences in microenvironmental cues (i.e., immune selective pressures and growth and ECM factor availability) could account for variations in estimated MMIC frequencies in NOD/SCID vis-à-vis NSG xenotransplantation experiments (Quintana et al. 2008; Schatton et al. 2008), they cannot explain apparent discrepancies in tumor-initiating ability of unfractionated melanoma cells assayed in the presence of matrigel and using identical mouse models (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Quintana et al. 2008). Specifically, the marked frequency of up to 1 in 4 unfractionated human melanoma cells with tumor-seeding ability in NSG hosts described by Quintana and colleagues (Quintana et al. 2010; Quintana et al. 2008) has not been confirmed to date. Indeed, independent laboratories have found that a minimum of 100 unfractionated patient-derived melanoma cells were required to consistently initiate tumors in NSG mice under equivalent experimental conditions (i.e., in the presence of matrigel) (Boiko et al. 2010; Boonyaratanakornkit et al. 2010), paralleling findings in additional CSC-driven malignancies, including pancreatic and head and neck cancers (Ishizawa et al. 2010). These new studies (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Ishizawa et al. 2010) alert us to the fact that MMICs might generally not be as common as suggested by Quintana et al. (2008, 2010). Thus, even experimental models that are very permissive to experimental

tumor growth support the view that only a minority of melanoma cells is capable of initiating and maintaining the disease.

How can the differences of the findings by Quintana and colleagues and other studies using apparently identical model systems (Boiko et al. 2010; Boonyaratanakornkit et al. 2010) be explained? For instance, variations in enzymatic tumor dissociation, isolation, and/or inoculation techniques between different laboratories could lead to different estimated MMIC frequencies (Shackleton 2010). Specifically, the aforementioned laboratory procedures could potentially account for differences in surface marker expression and/or viability among inoculated melanoma cell suspensions. Also, the use of distinct matrigel batches with varying growth and/or ECM factor compositions could result in altered melanoma growth. Alternatively, the use of tumor specimens from patients with more advanced disease (Quintana et al. 2008), which have been demonstrated to contain elevated CSC numbers compared to primary tumors (Schatton et al. 2008), might also account for higher relative proportions of melanoma cells capable of initiating experimental tumor growth. In addition, the assessment of CSC frequency, utilizing tumor cell isolates from melanoma xenografts that have been passaged *in vivo* for extended periods of time in lieu of directly patient-derived tumor samples, could further obscure the accurate assessment of CSC frequencies and biological functions (Boiko et al. 2010; Quintana et al. 2008). Indeed, Boiko and colleagues demonstrated that both *in vitro* and *in vivo* passaging of melanoma cells can result in the emergence of tumorigenic subclones independent of their immunophenotype (Boiko et al. 2010). Similarly, tumor cell lines may have lost the hierarchical structure of the primary tumor from which they originated (Zhou et al. 2009). A lesson to be learned from the apparently conflicting evidence regarding the frequency of tumorigenic melanoma cells in NSG mice (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Quintana et al. 2008) is that careful comparison of experimental methodologies may serve to explain differing results. Clearly, these discordant studies demonstrate that both the tumor environment and experimental procedures can govern tumor growth and underline the importance of establishing standardized assays for the study of MMIC biology that could enable the consolidation of experimental findings from different laboratories.

In summary, differences in MMIC frequency and function suggested by results obtained in alternative host immune milieus in the presence or absence of co-injected ECM and/or growth factors (Boiko et al. 2010; Quintana et al. 2008, 2010; Schatton et al. 2008) strongly suggest critical interactions of MMIC with the tumor host environment. Thus, bioassays and experimental model systems for the detection of clinically relevant MMICs require further approximation of, rather than further abstraction from, the naturally occurring tumor–host interactions in human patients (Frank et al. 2010; Schatton et al. 2009).

12.5

Melanoma Stem Biology: Therapeutic Opportunities

The ability to prospectively identify CSCs has permitted researchers to begin characterizing specific molecular and cellular mechanisms preferentially associated with CSCs that may contribute to tumor initiation and growth, in addition to those associated with their

defining features of unlimited self-renewal and proliferative capacities (Frank et al. 2010). Among the recently uncovered CSC functions likely to influence tumor development are mechanisms of tumor immune evasion (Schatton and Frank 2009). Findings of increased tumor incidence in immunocompromised patients suggest that immunosurveillance might serve to eliminate malignant cells at early stages of tumorigenesis (Mapara and Sykes 2004). Relative immune privilege and/or selective immunomodulatory functions could thus enable CSCs to evade antitumor immune responses in favor of inexorable tumor growth (Schatton and Frank 2009). This possibility is especially relevant to melanoma development, because melanoma is a particularly immunogenic cancer (Rosenberg et al. 2008). Indeed, several mechanisms by which MMICs evade antitumor immunity have recently been identified (Boiko et al. 2010; Schatton et al. 2010).

For example, MMICs can downregulate their expression of TAAs, such as MART-1, tyrosinase, ML-IAP, and the cancer testis antigens NY-ESO-1, and MAGE-A (Boiko et al. 2010; Schatton et al. 2010). Importantly, T cells reactive against TAAs are commonly observed in melanoma patients where they elicit antitumor immune responses directed at TAA-expressing melanoma cells (Lee et al. 1999; Stockert et al. 1998). Decreased expression of TAAs would thus enable MMICs to evade antitumor immune responses (Boiko et al. 2010; Schatton et al. 2010), providing for a potential explanation for the relative ineffectiveness of tumor-reactive T cells and autologous vaccination strategies in halting tumor growth (Schatton and Frank 2009). ABCB5⁺ MMICs were also found to express decreased levels of MHC class I molecules (Schatton et al. 2010), which represent an established mechanism of tumor immune evasion and neoplastic progression (Aptsiauri et al. 2007; Khong et al. 2004), further suggesting preferential MMIC evasion from immunological clearance.

In addition to reduced expression of MHC class I molecules, ABCB5⁺ MMICs selectively expressed the B7.2 (CD86) costimulatory ligand and the negative costimulatory receptor, PD-1 (Schatton et al. 2010). Of note, interactions of B7.2 with its receptor CTLA-4 and of PD-1 with its ligands PD-L1 and PD-L2 can downmodulate immune responses by inducing T cell anergy and/or by activating Tregs (Greenwald et al. 2005; Li et al. 2009a), suggesting that MMICs might regulate antitumor immune responses in favor of inexorable tumor growth via the involvement of negative costimulatory pathways. Indeed, selective blockade of MMIC-expressed B7.2 maintained CD4⁺CD25⁺FoxP3⁺ Treg frequencies among cocultured lymphocytes and regulated their secretion of the immunosuppressive cytokine IL-10 (Schatton et al. 2010). Importantly, accumulating evidence in various cancers, including melanoma, suggests that Tregs might represent important mediators of clinical tumor immune evasion (Ahmadzadeh et al. 2008; Clark et al. 2008; Curiel et al. 2004), underlining the potential importance of MMIC-mediated Treg induction for melanoma progression.

In addition, MMICs were also found to inhibit human peripheral blood mononuclear cell proliferation more efficiently than tumor bulk populations (Schatton et al. 2010). Consistently, ABCB5⁺ melanoma subpopulations preferentially inhibited production of the proliferative cytokine, IL-2, by both cocultured mitogen-activated lymphocytes and patient-identical peripheral blood mononuclear cells in the absence of a mitogenic stimulus (Schatton et al. 2010). Importantly, decreased IL-2 levels correlate with increased melanoma growth in animal models and human patients (Eklund and Kuzel 2004). Given its immune-activating effects, IL-2 is used as adjuvant therapy for the treatment of

advanced stage melanoma (Eklund and Kuzel 2004). Thus, preferential inhibition of IL-2 signaling might not only represent an important mechanism underlying MMIC-driven tumor growth but could also provide for a novel explanation for the commonly observed inability of therapeutic regimens involving IL-2 in producing durable patient responses (Eklund and Kuzel 2004). The preferential inhibition of IL-2 production by ABCB5⁺ MMICs might also explain observed differences in MMIC frequency in NSG (IL-2R $\gamma^{-/-}$) (Quintana et al. 2008) versus NOD/SCID (IL-2R^{WT}) hosts (Quintana et al. 2008; Schatton et al. 2008). Specifically, assessment of tumorigenicity outcomes in the absence of functional IL-2 signaling might overestimate MMIC frequency because a host environment characterized by abnormally impaired antitumor immunity might enable melanoma bulk populations to also contribute to tumor growth.

Additional immunomodulatory functions of MMICs include the secretion of immunosuppressive factors, such as immune-inhibitory TGF- β pathway members (Schatton et al. 2010). Importantly, these soluble mediators can be produced by tumors to dampen the anticancer immune response (Gorelik and Flavell 2001; Inge et al. 1992).

In sum, numerous MMIC-specific immunological mechanisms have been unraveled that may enable them to evade and/or modulate the antitumor immune response to promote neoplastic growth and progression. Strikingly, a relative immune privilege of CSCs was also demonstrated in alternative malignancies, including glioblastoma (Di Tomaso et al. 2010; Wei et al. 2010a, b), bladder cancer (Chan et al. 2009), colorectal carcinoma (Todaro et al. 2009), and leukemias (Majeti et al. 2009), indicating that immunomodulation might represent a common feature of CSCs (Schatton and Frank 2009). Clearly, the possibility of CSC-driven tumor immune escape has profound implications for the design of biologically relevant assays for the study of CSC behavior.

Perhaps, most significant are the implications of an MMIC-driven tumor immune escape for the development of improved cancer immunotherapeutic protocols. In addition, MMIC biology could provide a highly relevant tool for the evaluation of patient responses to current immunotherapeutic regimens, including those aimed at modulating critical regulatory elements of patient immune cells to enhance their antitumor reactivity. Importantly, a number of such novel immunotherapeutics have recently entered clinical trials (Kirkwood et al. 2008). These include inhibitors of costimulatory molecules or paracrine immunosuppressive cytokines, including monoclonal antibodies directed at CTLA-4, PD-1, 4-1BB, and TGF- β (Fong and Small 2008; Kirkwood et al. 2008; Lahn et al. 2005; Lynch, 2008). In contrast to current immunotherapeutic regimens, these novel agents exert inhibitory effects on both immune effector cells and Treg function (Kirkwood et al. 2008). They might thus prove more effective in inducing durable patient responses. Indeed, a recent phase III study demonstrated that treatment of stage III or IV melanoma patients with therapy-resistant disease with the anti-CTLA-4 antibody Ipilimumab resulted in improved overall survival compared to patients treated with a gp100 peptide vaccine (Hodi et al. 2010). A potential additional explanation for this encouraging antimeelanoma effect of CTLA-4 inhibition arises from the fact that tumorigenic MMICs preferentially expressed the CTLA-4 ligand, B7.2, and induced Tregs in a B7.2-dependent fashion (Schatton et al. 2010). Inhibition of MMIC-specific immune escape mechanisms might thus contribute to the antitumor efficacy of Ipilimumab. Given the preferential expression of PD-1, 4-1BBL, and TGF- β pathway

members by MMICs, it is further conceivable that responses observed in melanoma patients treated with anti-PD-1 antibodies (Brahmer et al. 2010), or therapeutic agents directed at 4-1BB (Lynch 2008) or TGF- β (Kirkwood et al. 2008), might potentially also relate to the ability of these immunotherapeutics to block MMIC-specific immunomodulatory functions. The observed failure of immunotherapies directed at the bulk of tumor cells (e.g., vaccination strategies targeting differentiation antigens such as MART-1 or tyrosinase) or those that elicit nonspecific immune activation (e.g., neoadjuvant IL-2 therapy) in mediating sustained patient responses (Rosenberg et al. 2004) might, on the other hand, relate to the inability of such regimens to effectively target the MMIC compartment (Schatton and Frank 2009), given its low expression levels of TAAs (Boiko et al. 2010; Schatton et al. 2010) and its preferential ability to inhibit IL-2 production (Schatton et al. 2010). Taken together, these findings highlight the possibility that immunotherapeutic strategies aimed at enhancing the endogenous immune responses to melanoma might prove most efficient if MMIC-specific immune escape mechanisms are concurrently impaired. In light of this intriguing possibility, it might be relevant to analyze the clinical effectiveness of novel immunotherapeutic agents, including Ipilimumab or PD-1 antibodies, not only with regard to the pattern and duration of immune responses (Reuben et al. 2006), but also in the context of their impact on the MMIC subset and its immunomodulatory properties (Schatton and Frank 2009).

In addition to their preferential refractoriness to current immunotherapeutic protocols, MMICs also demonstrate enhanced resistance to several structurally unrelated chemotherapeutic agents with distinct mechanisms of action (Schatton et al. 2009), a phenomenon also termed multidrug resistance (MDR) (Dean et al. 2005). MDR can result through several different means, including impairment of tumor apoptotic pathways, alterations in cell cycle checkpoints, and decreased drug accumulation (Gottesman et al. 2002). The latter mechanism is of particular interest to MMICs, because one mechanism resulting in reduced intracellular drug levels is the excretion of cytotoxic agents by energy-dependent efflux pumps, known as ATP-binding cassette (ABC) transporters (Gottesman et al. 2002). Specifically, the MMIC determinant, ABCB5 (Schatton et al. 2008), mediates resistance to multiple chemotherapeutic agents in malignant melanoma, hepatocellular carcinoma, breast cancer, and leukemias, including doxorubicin (Cheung et al. 2011; Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Yang et al. 2010), camptothecin, mitoxantrone, and 5-fluorouracil (Huang et al. 2004; Lehne et al. 2009). A broader role for the ABCB5 transporter in chemotherapeutic resistance to additional agents is suggested by the observation that ABCB5 mRNA expression levels across a panel of human cancer cell lines used by the National Cancer Institute for drug screening correlated significantly with chemoresistance to 45 out of 119 anticancer agents (Frank et al. 2005). Evidence for a preferential resistance of CSCs to both chemotherapy and radiotherapy has also been generated in numerous additional CSC-driven malignancies, including leukemias, gliomas, breast carcinomas, and pancreatic cancer (reviewed in (Schatton et al. 2009)), underscoring the need to dissect further the molecular pathways responsible for CSC-specific therapy resistance. Furthermore, they highlight the therapeutic promise of MMIC-directed treatment strategies, which could enhance current treatment modalities for patients with advanced stage melanoma.

This possibility is further highlighted by recent findings of a relationship of MMICs to neoplastic melanoma progression (Fukunaga-Kalabis et al. 2010; Gazzaniga et al. 2010; Ma et al. 2010; Schatton et al. 2008; Sharma et al. 2010; Vasquez-Moctezuma et al. 2010) and adverse clinical outcome (Sharma et al. 2010). In light of the CSC concept and findings of MMIC-specific immunoregulatory properties, a number of novel therapeutic approaches can be envisioned that could potentially increase the efficacy of current forms of therapy (reviewed in (Frank et al. 2010)), if MMICs are indeed the major culprits of melanoma initiation and clinical virulence. For instance, MMIC ablation through prospective markers might prove useful in enhancing current antimelanoma therapies. In support of this possibility, selective killing of MMICs via ADCC using an anti-ABCB5 monoclonal antibody halted experimental tumor growth (Schatton et al. 2008). Alternatively, targeting of MMICs via preferentially expressed surface molecules and/or MMIC-specific pathway interference might also yield improved melanoma patient responses. Such treatment strategies could include inhibition of costimulatory signaling events mediated by MMIC-expressed (Schatton et al. 2010) B7.2, PD-1, and/or 4-1BB. Disruption of surrogate MMIC-specific immune evasion pathways could also represent treatment modalities that might enhance responsiveness to current antimelanoma regimens. In addition, driving MMICs into differentiation could lead to improved therapeutic outcomes. Consistent with this possibility, differentiation of glioblastoma CSCs via BMP4 (bone morphogenetic protein 4) exposure resulted in inhibition of experimental tumor growth concomitant with enhanced survival (Piccirillo et al. 2006). Interestingly, the BMP4 receptor, BMPRIa, is preferentially expressed by ABCB5⁺ MMICs (Schatton et al. 2008), suggesting that a similar strategy could also be promising in this malignancy. An additional MMIC-directed targeting strategy could involve the use of antiangiogenic and/or antivasculogenic regimens. This possibility is indicated by findings of a preferential induction of neovascularization by CSCs in other cancers (Bao et al. 2006b). MMICs might likewise be involved in angiogenesis and/or vasculogenesis given their preferential expression of vasculogenic differentiation markers VE-cadherin and TIE-1 (Schatton et al. 2008). Reversal of chemoresistance and/or radioresistance mechanisms operative in MMICs might also successfully increase antimelanoma therapeutic efficacy (Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Huang et al. 2004). This is further suggested by findings in glioblastoma CSCs (Bao et al. 2006a), among other cancers (Diehn et al. 2009; Vlashi et al. 2009). Lastly, given the importance of the tumor environment in governing CSC behavior (Scadden 2006), the disruption of protumorigenic MMIC-niche interactions could also optimize anticancer therapeutic protocols. It is important to recognize that therapeutic efficacy will often depend also on a significant reduction of melanoma bulk populations, which may cause excessive tumor burden. Therefore, combination therapies that involve both MMIC-directed agents as well as debulking regimens would be predicted to prove most effective in improving clinical treatment responses and patient outcomes.

In summary, several biological functions of MMICs have recently been identified, including their preferential ability to modulate and/or evade antitumor immune responses (Boiko et al. 2010; Schatton et al. 2010). In addition to their refractoriness to current immunotherapeutic protocols, MMICs also demonstrate increased resistance to a number of chemotherapy agents (Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Huang et al. 2004). Taken together with the recently established relationship of

MMICs to melanocytic tumor progression (Fukunaga-Kalabis et al. 2010; Gazzaniga et al. 2010; Ma et al. 2010; Schatton et al. 2008; Sharma et al. 2010; Vasquez-Moctezuma et al. 2010), these findings underscore the potential clinical relevance of MMIC-directed targeting approaches. Importantly, proof-of-principle for the potential therapeutic utility of targeting MMICs has been established, by demonstrating that selective eradication of ABCB5⁺ melanoma subpopulations can halt experimental tumorigenesis (Schatton et al. 2008). A number of additional targeting approaches might likewise prove useful in blocking MMIC-driven tumor development and progression, including disruption of immune-inhibitory pathways, differentiation therapy, and chemoresistance reversal. While more work is required to translate these research developments into the clinic, they point to a critical relevance of MMICs for successful melanoma therapy. Further molecular screens of purified MMIC populations employing microarrays, RNA-interference, or drug-screening libraries could allow identification of further targetable pathways leading to MMIC-tailored eradication strategies for melanoma therapy.

12.6

Conclusions

A growing body of literature supports the existence of MMICs (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Schatton et al. 2008). Additionally, important links have been established between MMICs, neoplastic progression (Fukunaga-Kalabis et al. 2010; Gazzaniga et al. 2010; Ma et al. 2010; Schatton et al. 2008; Sharma et al. 2010; Vasquez-Moctezuma et al. 2010), chemoresistance (Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Huang et al. 2004), and refractoriness to immunotherapy (Boiko et al. 2010; Schatton et al. 2010). Moreover, novel biological features of MMICs are currently being intensively explored. For instance, the ability of MMICs to modulate the antitumor immune response (Schatton and Frank 2009; Schatton et al. 2008) might be especially informative for the optimization and/or evaluation of targeted immunotherapies that were recently found to improve overall survival in patients with recurrent metastatic melanoma (Hodi et al. 2010). Together, these findings highlight the relevance and promise of novel MMIC-centered diagnostic and therapeutic approaches in human melanoma.

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Abstract The plastic phenotype of aggressive melanoma has presented a significant challenge in the detection and targeting of tumor cells exhibiting stem cell-like characteristics. As the molecular signaling pathways underlying tumor cell plasticity become more transparent, our understanding of how to suppress this elusive phenotype will be enhanced. Indeed, we are making progress in identifying critical embryonic pathways, such as the Nodal signaling pathway, that reemerge in aggressive tumor cells – in the absence of regulatory check points. Because Nodal is not expressed by the majority of normal adult tissues, and is over-expressed by aggressive tumor cells, it represents a valuable new therapeutic target. Collectively, we have learned a great deal from studies that focus our attention on the convergence of embryonic and tumorigenic signaling pathways. At this interaction of normal development and tumor formation reside the clues to suppressing cancer progression.

13.1

Reemergence of an Embryonic Phenotype

Aggressive tumor cells express a plastic, multipotent phenotype similar to embryonic stem cells. However, the absence of major regulatory checkpoints in the tumor cells allows aberrant activation of embryonic signaling pathways, which appears to contribute to their plastic phenotype and aggressive behavior. For example, studies detailing the molecular signature of melanoma cells have revealed that aggressive tumor cells express genes (and proteins) associated with various cell types (including progenitor cells), while simultaneously downregulating genes specific to their melanocytic lineage (Bittner et al. 2000; Seftor et al. 2002). Particularly noteworthy are the phenotypes associated with endothelial cells and human embryonic stem cells (hESCs) that are expressed by human aggressive

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melanoma cells (reviewed in Hendrix et al. 2003a; Postovit et al. 2007; Hendrix et al. 2007; Schatton and Frank 2008). In melanoma, the endothelial phenotype (expressing vascular endothelial cadherin [VE-cadherin]) can engage in vasculogenic mimicry, which contributes to the formation of an extravascular fluid-conducting network in aggressive tumors (Fig. 13.2) coincident with a poor clinical outcome (Maniotis et al. 1999; Hendrix et al. 2001; Hendrix et al. 2003b; Demou and Hendrix 2008). It is important to note that vasculogenic mimicry represents an alternative vascularization mechanism in cancer that can accompany vessel co-option of preexisting vessels, mosaic vessels, angiogenesis, vasculogenesis, and intussusceptive microvascular growth (reviewed by Dome et al. 2007). Moreover, experimental studies have shown the plastic, transendothelial functionality of human metastatic melanoma cells as they express anticoagulant factors relevant for perfusion (Ruf et al. 2003) and participate in the neovascularization of circulation-deficient tissues (Hendrix et al. 2002). Vasculogenic mimicry is one example of the plastic phenotype associated with aggressive behavior in human melanoma, and it is noteworthy to mention that this endothelial-like phenotype has been reported in many tumor types, including breast carcinoma, prostatic carcinoma, ovarian carcinoma, synoviosarcoma, Ewings sarcoma, rhabdomyosarcoma, pheochromocytoma, and glioblastoma (reviewed by Hendrix et al. 2003a; Paulis et al. 2010; El Hallani et al. 2010). Interestingly, during normal development, cytotrophoblasts engage in vasculogenic mimicry during the formation of the placenta (Zhou et al. 1997). Therefore, the formation of a vascular perfusion pathway in aggressive tumors appears to recapitulate an early developmental event.

13.1.1

Nodal Signaling in Melanoma

A key embryonic pathway that is reactivated in melanoma and contributes to the stem cell-like phenotype, vasculogenic mimicry (Fig. 13.1), and aggressive behavior is Nodal signaling. Nodal is a member of the TGF- β superfamily and a critical embryonic morphogen and regulator of cell fate, particularly important in maintaining pluripotency in hESCs (Schier and Shen 2000; Schier 2003; James et al. 2005; Vallier et al. 2009). Nodal propagates its signal by binding to heterodimeric complexes of type I (ALK 4/7) and type II (ActRIIB) activin-like kinase receptors, which leads to the phosphorylation and activation of ALK 4/7 by ActRIIB and subsequent ALK 4/7-mediated phosphorylation of Smad-2 and Smad-3 (Yeo and Whitman 2001). Phosphorylated Smad-2/3 then associates with Smad-4, which translocates to the nucleus where it can regulate gene expression via association with specific transcription factors (Schier 2003). Nodal can signal directly with (or independently of) its co-receptor, Cripto-1, a member of the EGF Cripto-1/FRL-1/Cryptic (CFC) cell family (Yeo and Whitman 2001; Strizzi et al. 2005). A potent morphogen like Nodal requires tight regulatory control of its biological activity, which is critical in the initiation of mesoderm formation as well as in left-right patterning in the embryo. One of the most powerful inhibitors controlling Nodal signaling by spatially and temporally restricting the Nodal-mediated activation of ALK 4/7 is Lefty. More specifically, Lefty A and Lefty B are highly divergent members of the TGF- β superfamily that antagonize the Nodal signaling pathway by binding directly to Nodal, or by binding to Cripto-1 and pre-

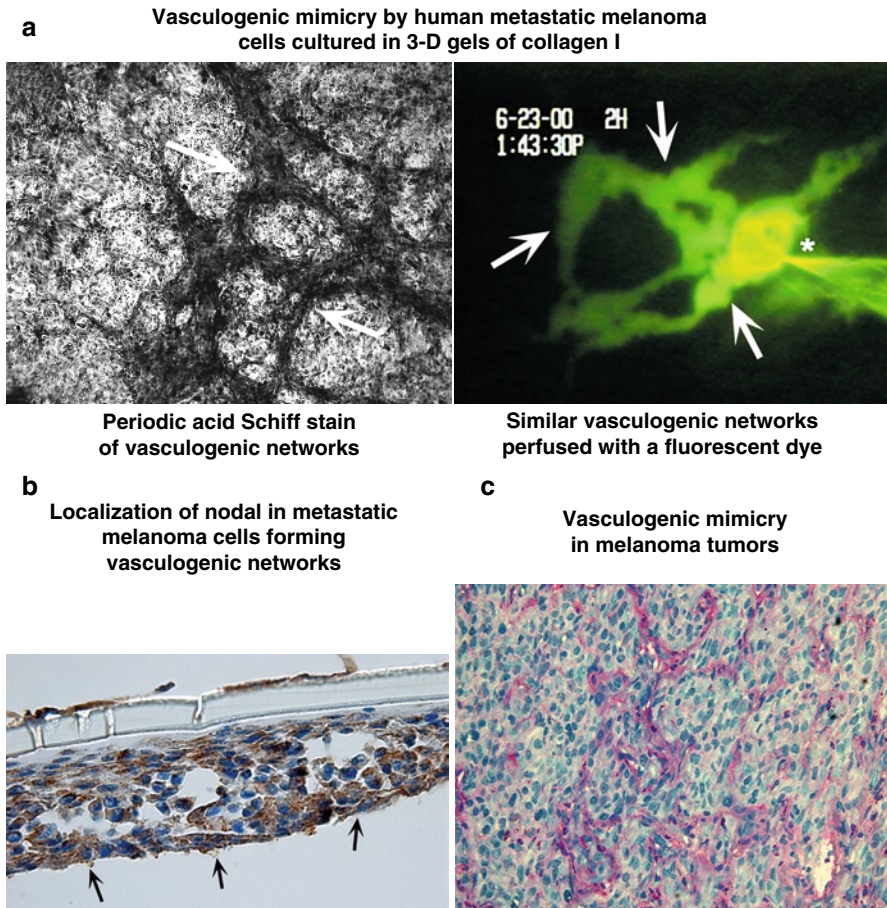
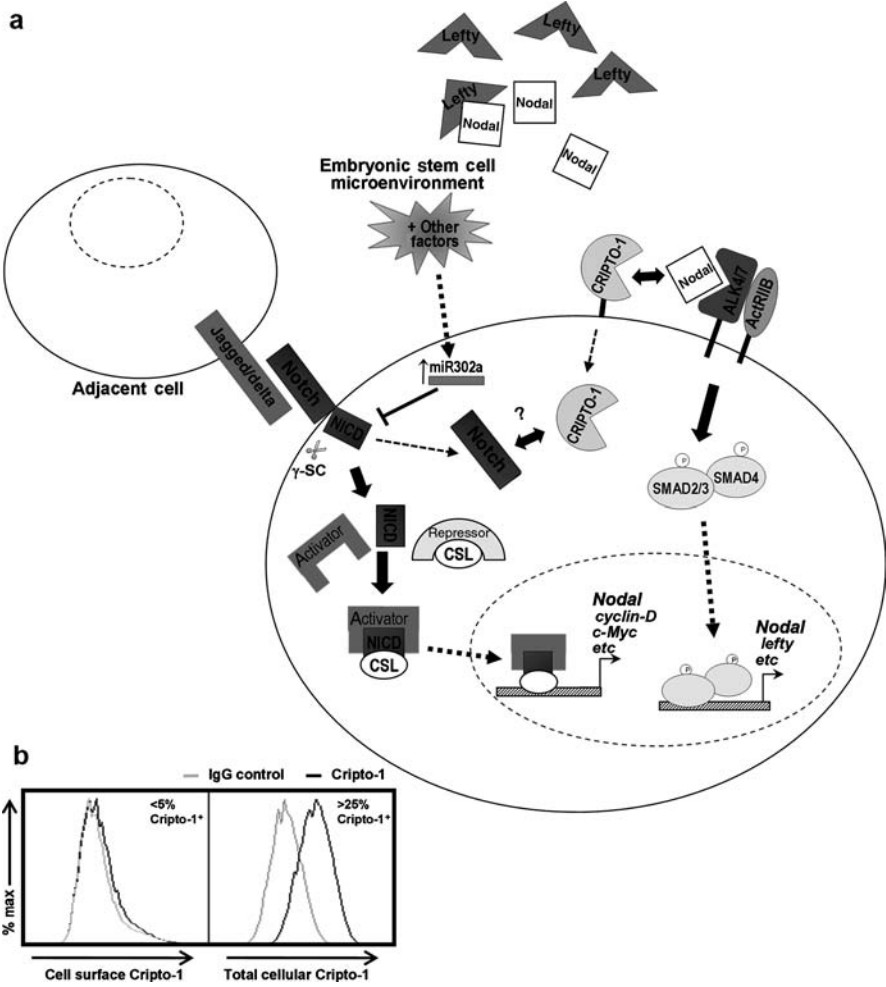


Fig. 13.1 Formation of vasculogenic networks by aggressive melanoma cells *in vitro* and *in vivo*. (a) Aggressive melanoma cells form vasculogenic networks (left; arrows) in 3-D gels of collagen I *in vitro*. Networks begin to form by the end of day 1 and continue to mature into vasculogenic structures (comprised of laminin and collagen; Seftor et al. 2001) by day 3 (left; periodic acid Schiff stained to help highlight networks). These network structures (arrows) become perfusable (with a fluorescent dye; “*” denotes point of injection) by day 14 (right). (b) Immunohistochemical localization of Nodal in a cross-section of a 7-day-old culture of metastatic melanoma cells forming vasculogenic networks in 3-D collagen I gels (on a polycarbonate membrane). [These findings have been validated *in vivo* by McAllister et al. 2010 using in situ hybridization for Nodal detection.] (c) PAS (Periodic Acid Schiff)-staining (pink) of vasculogenic mimicry networks in a histological section from a patient’s melanoma tumor (Original magnifications: (a) 10 \times , left; 25 \times , right; (b) 63 \times ; (c) 40 \times)

venting Nodal from forming a more active signaling complex with the type I and II activin receptors (Chen and Shen 2004). This regulation of Nodal expression and signaling can occur in the extracellular microenvironment where Nodal and Cripto-1 may be present, as well as at the cell surface (Fig. 13.2).

Recent findings have revealed the reactivation of embryonic Nodal signaling in melanoma (Topczewska et al. 2006; Postovit et al. 2008a, b; Yu et al. 2010). Compared to poorly aggressive melanoma cells that do not typically express detectable levels of Nodal, aggressive melanoma cells express Nodal mRNA and protein that contributes to their plastic, tumorigenic, and metastatic phenotype (Topczewska et al. 2006; Postovit et al. 2008a, b). Both hESCs and aggressive melanoma cells express robust levels of Nodal, and no point mutations in this gene have been detected in the tumor cells. Most noteworthy is the observation that unlike hESCs, aggressive melanoma cells do not express Lefty, and therefore, Nodal signaling in tumor cells occurs in a highly unregulated manner. Further analysis of Lefty in these tumor cells has revealed that the gene is methylated, suggesting a key underlying mechanism for silencing its expression (Costa et al. 2009). Nodal signals via a Smad-2-dependent positive feedback loop (Topczewska et al. 2006),



as a TGF- β family member. Indeed, the significance of Nodal underlying the plastic and tumorigenic phenotype of aggressive melanoma was demonstrated by downregulating its expression in these tumor cells, which resulted in the suppression of VE-cadherin (the endothelial-like phenotype) and the reexpression of Tyrosinase (the melanocytic phenotype), accompanied by the inhibition of tumorigenicity (Topczewska et al. 2006). Initial assessment of Nodal as a possible biomarker for melanoma disease progression in patients indicates that Nodal is associated with the aggressive melanoma phenotype expressing stem cell-like characteristics (Strizzi et al. 2009a; Yu et al. 2010).

13.1.2

Notch Signaling in Melanoma and Implications for Cripto-1

Across species, Notch signaling is a key pathway in embryogenesis, and, like Nodal, is involved in stem cell maintenance and cell fate determination as well as organ development and homeostasis (Chiba 2006; Bolós et al. 2007) (see also Chap. 5). Importantly, Notch signaling also contributes to melanoma development and metastatic behavior. The Notch pathway requires contact between neighboring cells for signal activation as both receptor and ligand are membrane bound proteins. Notch receptor activation results in cleavage of the intracellular domain that acts in a transcription factor complex to promote gene expression. Global suppression of intracellular domain cleavage by gamma-secretase inhibitors promotes melanoma cell apoptosis and decreases tumor volume *in vivo* in a xenograft nude mouse model, yet does not influence melanocyte cell survival, indicating a general role for Notch signaling in melanoma (Qin et al. 2004; Nickoloff et al. 2005). While the mammalian Notch receptor family has four members (Notch1–4), only Notch1 and Notch4 have so far been specifically linked to melanoma. Notch1 functions predominantly in the early stages of melanoma development, and can transform human melanocytes and early stage melanoma cells to a more aggressive phenotype, but does not appear

Fig. 13.2 Cross-talk between the Notch and Nodal signaling pathways. **(a)** The binding of Nodal to Type I and Type II Activin-like kinase receptors (ALK4/7 and ActRIIB, respectively) activates the second messengers, Smad-2/3 and Smad-4 in a Cripto-1-dependent or Cripto-1-independent manner. Phosphorylated Smad-2/3/4 translocates to the nucleus to promote the transcription of genes including *Nodal* itself, and in the context of embryonic development Nodal's antagonist, *Lefty*. Notch receptor binding to a Jagged or Delta ligand on an adjacent cell promotes the cleavage of the Notch intracellular domain (NICD) into the cytoplasm by gamma-secretase (γ -SC). Cleaved NICD binds co-activator proteins such as mastermind-like protein (activator) and the CSL DNA-binding protein to activate a nuclear gene transcription program that can include *Cyclin-D* and *c-Myc*, and specifically in the case of Notch4 in metastatic melanoma, *Nodal* gene expression. In the absence of cytoplasmic NICD, CSL binds co-repressor proteins (repressor). It is possible that Notch may be further regulated by interactions with intracellular Cripto-1. Furthermore, the upregulation of microRNAs such as miR-302a, induced by factors in the embryonic stem cell microenvironment, may provide an additional level of regulation of Notch receptor expression. **(b)** By flow cytometry fewer than 5% of C8161 melanoma cells express Cripto-1 on the cell surface (*left panel*), while greater than 25% of cells express some Cripto-1 (*right panel*), indicating that many cells harbor intracellular Cripto-1 protein in the absence of protein at the cell surface. *Gray lines* indicate IgG-treated negative controls; *black lines* indicate Cripto-1-stained cells

to affect metastatic cell behavior (Balint et al. 2005; Liu et al. 2006; Bedogni et al. 2008; Pinnix et al. 2009). Notch4, however, correlates with advanced stage disease progression and functions to regulate aggressive melanoma cell behavior (Hardy et al. 2010).

Early in mammalian development, during the establishment of left-right asymmetry, Notch signaling directly regulates expression of the Nodal gene and this interaction is required for subsequent organ positioning and normal development (Krebs et al. 2003; Raya et al. 2003). A similar relationship between the Notch and Nodal pathways is reestablished in metastatic melanoma (Fig. 13.2), and this molecular cross-talk is suggested via the activity of a CSL-dependent Nodal enhancer element (Hardy et al. 2010). Of importance, Notch4 and Nodal expression correlate specifically in aggressive melanoma cells, and co-expression of proteins is observed in a subset of the cell population (Hardy et al. 2010). In fact, Notch4 lies upstream of Nodal in aggressive cells, as downregulation of Notch4 expression or antagonism of receptor activity with neutralizing antibodies depletes expression of Nodal, while over-expression of a truncated, activated form of Notch4 can upregulate Nodal expression in depleted aggressive cells or in poorly aggressive cells. Coincident with this, Notch4 activity influences the aggressive behavior of metastatic cells, at least partially through its regulation of Nodal expression, as antagonism of Notch4 receptor activity reduces vasculogenic mimicry and anchorage-independent growth *in vitro*, and this can be rescued by treatment with recombinant human Nodal protein. It is clear that cross-talk between Notch and Nodal signaling is an important component of the aggressive melanoma phenotype, and offers an attractive potential target for melanoma therapy.

The co-receptor for Nodal, Cripto-1, also has essential functions in embryogenesis, but is less well characterized in melanoma. C8161 melanoma cells express low levels of Cripto-1 (Postovit et al. 2008a), but nonetheless display a distinct expression pattern of Cripto-1 protein. On the cell surface, where it serves as a co-receptor for Nodal, Cripto-1 can be detected by flow cytometry on fewer than 5% of C8161 cells, whereas greater than 25% of cells harbor intracellular Cripto-1 (Fig. 13.2b). Interestingly, C8161 cells that express surface Cripto-1 can give rise to non-adherent spheres of cells in culture that harbor increased levels of Nanog and OCT4 proteins; both transcription factors know to modulate ES cell self-renewal and pluripotency (Strizzi et al. 2008). In fact, Cripto-1 is a direct target of OCT4- and Nanog-mediated transcription in ES cells, consistent with its early function in maintaining pluripotency (Loh et al. 2006). Whether Cripto-1 can be considered a marker for melanoma “stem-like” cells remains to be validated, but it is tempting to speculate that expression of Cripto-1 by melanoma cells may hold clues to the integration of Nodal and Notch signaling in the population of plastic melanoma “stem-like” cells as they respond to a changing microenvironment (Fig. 13.2). Recent studies demonstrate that Nodal can signal by Cripto-1-dependent and Cripto-1-independent mechanisms (Liguori et al. 2008); however, the particular contribution of Cripto-1 to Nodal signaling and to the specific outcomes of Nodal signaling in melanoma remains unclear. Finally, inside the cell, Cripto-1 has been shown to bind all four Notch receptors and promote their maturation and signaling (Watanabe et al. 2009), suggesting that Cripto-1 may function in the complex integration of numerous signaling pathways that regulate phenotype switching and plasticity in melanoma “stem-like” cells.

13.2

Reprogramming the Metastatic Phenotype

13.2.1

Human Embryonic Stem Cell Microenvironment

Embryonic stem cells sustain a microenvironment that facilitates a balance of self-renewal and differentiation. Aggressive cancer cells, expressing a multipotent, embryonic cell-like phenotype, engage in a dynamic reciprocity with a microenvironment that promotes plasticity and tumorigenicity (see also Chap. 14). However, the cancer associated milieu lacks the appropriate regulatory mechanisms to maintain a normal cellular phenotype. Based on the aberrant expression of the Nodal plasticity gene by melanoma tumor cells, an experimental study tested whether these cells could respond to regulatory cues controlling the Nodal signaling pathway, which might be sequestered within the microenvironment of hESCs, resulting in the suppression of the tumorigenic phenotype (Postovit et al. 2006) (Fig. 13.3). Specifically, it was discovered that exposure of the tumor cells to a hESC microenvironment (containing Lefty) leads to a dramatic downregulation of their Nodal expression concomitant with a reduction in clonogenicity and tumorigenesis accompanied by an increase in apoptosis. Furthermore, this ability to suppress the tumorigenic phenotype is directly associated with the secretion of Lefty, exclusive to hESCs, because it is not detected in other stem cell types or trophoblasts. The tumor-suppressive effects of the hESC microenvironment, by neutralizing the expression of Nodal in aggressive tumor cells, provide previously unexplored therapeutic modalities for cancer treatment. Further analysis of the possible mechanisms underlying the epigenetic reprogramming of aggressive melanoma cells exposed to the hESC microenvironment used bisulfite-sequencing technology and discovered a marked increase in site-specific methylation in the Nodal CpG island – a 32% increase in DNA methylation and concomitant silencing of the Nodal gene (Postovit et al. 2007). Assessment of differential microRNA (miRNA) expression revealed the upregulation of miR-302a in melanoma cells exposed to the hESC microenvironment (Costa et al. 2009). Interestingly, Notch4, another stem cell marker associated with endothelial progenitors, is a target of miR-302a. These findings provided new insights into the epigenetic changes such as DNA methylation and regulation by microRNAs that might play a significant role in tumor cell plasticity and the metastatic phenotype.

13.2.2

Neural Crest Microenvironment

Recently identified commonalities between tumor cell behaviors and normal embryonic developmental pathways suggest that some tumor types derive certain traits from their ancestral cell of origin. One such example is melanoma. Pre-neoplastic melanocytes derive from a highly invasive and multipotent embryonic cell population called the neural crest

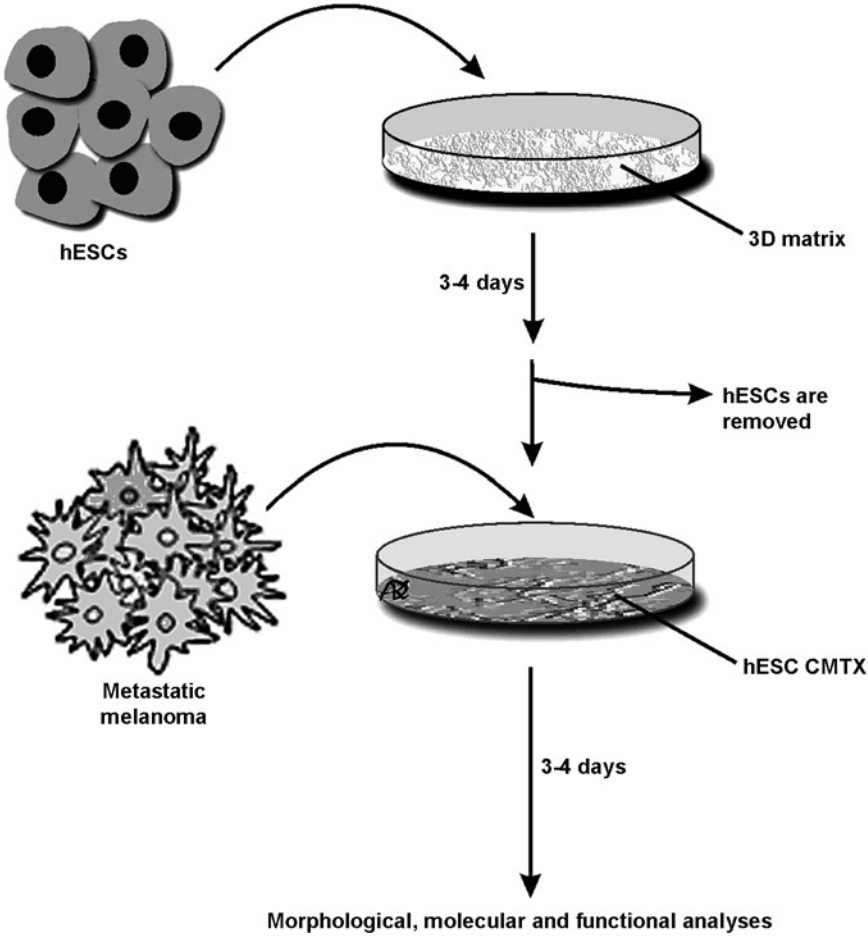


Fig. 13.3 Human embryonic stem cell microenvironment. Compact colonies of human embryonic stem cells (hESCs) are seeded onto a 3-D matrix for 3–4 days, then removed, resulting in a conditioned 3-D matrix (CMTX) onto which human metastatic melanoma cells are then seeded and incubated for 3–4 days. Subsequently, changes in cell morphology, gene and protein expression as well as the behavioral function(s) of the tumor cells can be examined

(NC). The vertebrate embryo regulates the programmed invasion of the NC such that cells migrate in discrete streams along stereotypical migratory pathways. Importantly, the NC microenvironment contains signals that not only guide NC migration but also regulate fate determination. Recent work has focused on harnessing this unique microenvironment to study melanoma cell behaviors *in vivo* (Fig. 13.4). By transplanting human melanoma cells into the chick NC microenvironment, melanoma cells have been shown to respond to host NC cues and emigrate along NC migratory routes in both the head and trunk. Furthermore, the NC microenvironment appears to possess the potential to control and revert the metastatic phenotype.

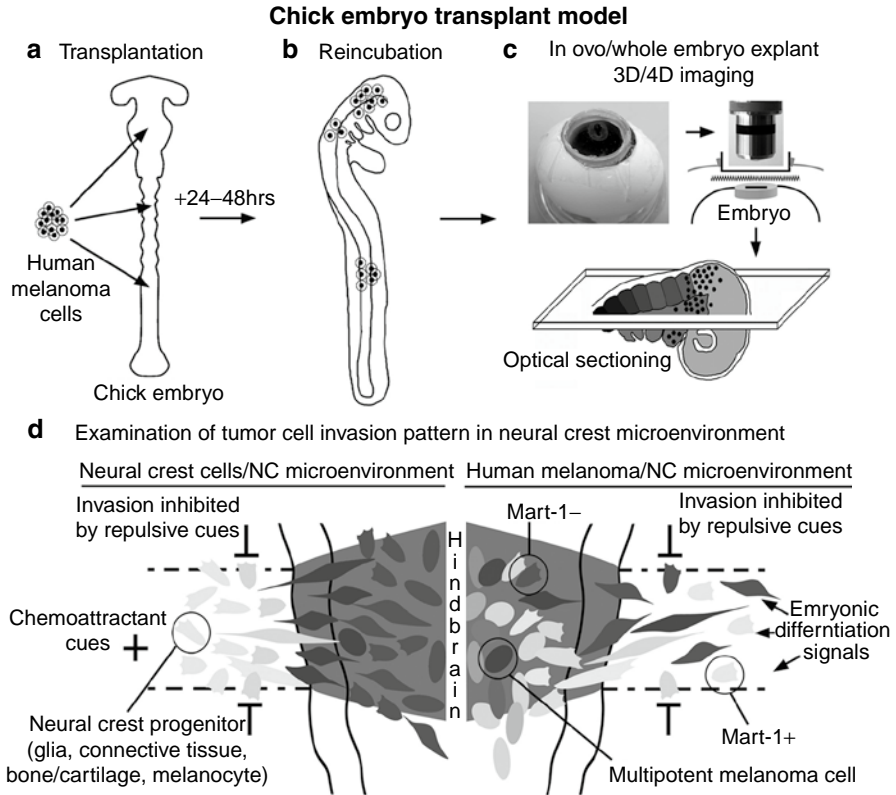


Fig. 13.4 A schematic diagram of the chick embryo transplant model. **(a)** Human melanoma cells are transplanted into the embryonic neural crest microenvironment at different axial levels during early stages of chick development. **(b)** Eggs are re-incubated at 37°C to allow tumor cells to interact and emigrate onto the host chick embryonic neural crest migratory pathways. **(c)** Eggs are then either prepared for in ovo 4-D time-lapse imaging through a window in the eggshell or whole embryo explants are prepared for 3-D optical sectional to visualize tumor cell positions, using confocal or two-photon microscopy. **(d)** The tumor cell invasion pattern (*right-hand side*) is analyzed and compared to the host chick neural crest cell migration pattern (*left-hand side*) shown here at the level of the chick hindbrain, the site of the transplant. Multipotent neural crest cells are sculpted onto stereotypical migratory pathways by a combination of intrinsic and extrinsic cues, including inhibitory and chemotactic guidance signals that maintain cells in an ordered, programmed invasion pattern. Multipotent, metastatic melanoma cells placed into the chick embryonic neural crest microenvironment respond to local signals and follow host neural crest migratory pathways. A subset of metastatic melanoma cells that invade into the embryo express MART-1, a gene that is not expressed prior to transplantation into the chick embryonic neural crest microenvironment, suggesting local signals reprogram the cell toward a melanocyte-like phenotype

Recent studies have discovered several candidate molecules that influence NC cell migratory behaviors (Kuriyama and Mayor 2008; Kulesa et al. 2010; Gammill and Roffers-Agarwal 2010). For example, neuropilin signaling has been shown to play a critical role in NC cell pathfinding (Kulesa et al. 2010; Gammill and Roffers-Agarwal

2010). In the head, neuropilin signaling appears to influence the position of early migratory NC cells near the hindbrain (Roffers-Agarwal and Gammill 2009), and facilitate the chemotactic invasion of NC cells into precise target sites, through neuropilin–VEGF interactions (McLennan and Kulesa 2007; McLennan and Kulesa 2010; McLennan et al. 2009). There is also *in vivo* evidence for the chemokine receptor CXCR4 and its ligand, SDF-1, to act as a chemoattractant that guides the migration of trunk NC cells to the sympathetic ganglia to become sympathetic neurons (Kasemeier-Kulesa et al. 2010). Thus, what has clearly emerged is a picture whereby signals within the NC microenvironment are coordinated in space and time to produce the NC cell migratory pattern (Kulesa and Gammill 2010).

Observations of the plastic phenotype of melanoma cells in an experimental model of the NC has led to a hypothesis that metastatic melanoma cells could respond to an embryonic microenvironment experienced by their ancestral NC cell type. To test whether human metastatic melanoma cells transplanted into the NC microenvironment would follow host migratory pathways, the invasive behavior of tumor cells was examined in a chick embryo transplant model (Fig. 13.4). These studies found a subset of transplanted GFP-labeled metastatic melanoma cells (C8161) that invaded the chick periphery along host NC cell migratory pathways and did not reform tumors (Kulesa et al. 2006). In contrast, the poorly metastatic melanoma cells (C81-61) did not emigrate from transplant sites (Kulesa et al. 2006). To test whether human metastatic melanoma cells transplanted into the chick NC microenvironment would express phenotype-specific genes associated with the acquisition of a neural crest melanocyte-like phenotype, chick embryos were examined for the immunolocalization of MART-1, a melanocyte differentiation antigen that is commonly lost during neoplastic transformation (Fig. 13.4). Using this marker, a subset of transplanted GFP-labeled melanoma cells was found that invades the chick periphery along NC migratory pathways and did express MART-1 suggesting that signals within the NC microenvironment could revert the phenotype of metastatic melanoma cells (Kulesa et al. 2006).

A probable link between NC cell induction and guidance cues within the chick microenvironment and the potential roles of these molecular signaling pathways in the development of malignant melanoma can be observed when comparing highly aggressive versus poorly aggressive human melanoma cells (Kasemeier-Kulesa et al. 2008). These experiments have revealed that some guidance signals that define NC cell migratory pathways are upregulated in malignant melanoma cells. For example, neuropilin/semaphorin (neuropilin-1 is upregulated 28-fold) and Eph/ephrin (EphA4 is upregulated 4.2-fold) signaling pathways are well-established NC cell guidance pathways (Kulesa and Gammill 2010). Yet little is known about these pathways in the involvement of melanoma invasion and metastasis. To better understand how malignant melanoma cells may usurp NC cell induction and guidance pathways to promote metastasis *in vivo*, a gene profiling approach, in combination with the chick embryo transplant model is being employed to delineate the expression of genes implicated in both NC cell migration and melanoma metastasis (Fig. 13.4). Preliminary results suggest that there is a causal link between the steps of NC cell specification, delamination, and migration and those involved in melanoma metastasis.

In summary, recent evidence highlighted here has revealed an important linkage underlying the convergence of embryonic and tumorigenic signaling, within the chick embryonic NC microenvironment. With emerging evidence for NC cell chemotaxis and tools for molecular profiling, it will be exciting to test whether metastatic melanoma cells transplanted into the embryonic NC microenvironment adopt a signature common to the host NC or selectively regulate genes to take advantage of local guidance information. Thus, the use of the chick embryonic NC microenvironment model to study tumor cell migration and phenotype determination offers an innovative approach to investigate tumor cell plasticity and metastasis *in vivo*, and the potential for tumor cell reprogramming by identifying and targeting the convergence of embryonic and tumorigenic signaling pathways.

13.3

Targeting the Embryonic Phenotype to Suppress Metastasis

Therapeutic strategies that target a vascular supply to growing tumors and/or specific tumor cell surface antigens have been employed as conventional approaches over the past several decades. However, the most aggressive tumor cell phenotype, as is the case with metastatic melanoma, poses a significant challenge with respect to drug resistance and disease recurrence. The basic discoveries presented in this chapter focus on the reexpression of an embryonic phenotype by aggressive melanoma cells that enable them to remain resistant against classical angiogenesis inhibitors such as Endostatin (Fig. 13.5). Specifically, studies have shown that when Endostatin was administered to metastatic melanoma or microvascular endothelial cells in 3-D culture, angiogenesis was inhibited in the endothelial cells, but vasculogenic mimicry in melanoma cells was not suppressed (van der Schaft et al. 2004). The molecular mechanism(s) underlying this differential response was based on the robust expression of the Endostatin receptor, α_5 -integrin, by the endothelial cells, which was not found in sufficient levels in melanoma cells. These important findings hopefully provide novel insights into the development of new anti-vascular therapeutic agents that could target both angiogenesis and tumor cell vasculogenic mimicry.

Since the introduction of vasculogenic mimicry to the cancer research community (Maniotis et al. 1999), an impressive body of evidence has emerged in numerous laboratories highlighting the signaling pathways underlying vasculogenic mimicry (Hess et al. 2007; Paulis et al. 2010), which has provided valuable new targets for the development of rational treatment modalities. Indeed, the recent findings showing the significance of Nodal reexpression directly contributing to plasticity and tumorigenicity illuminate this dysregulated embryonic pathway as a promising new target in melanoma therapy, and possibly other cancers as well. These basic findings have been further advanced in preclinical melanoma models by using function-blocking Nodal antibodies to suppress vasculogenic mimicry and inhibit experimental metastasis *in vivo* (Strizzi et al. 2009a). The most recent discovery demonstrating the molecular

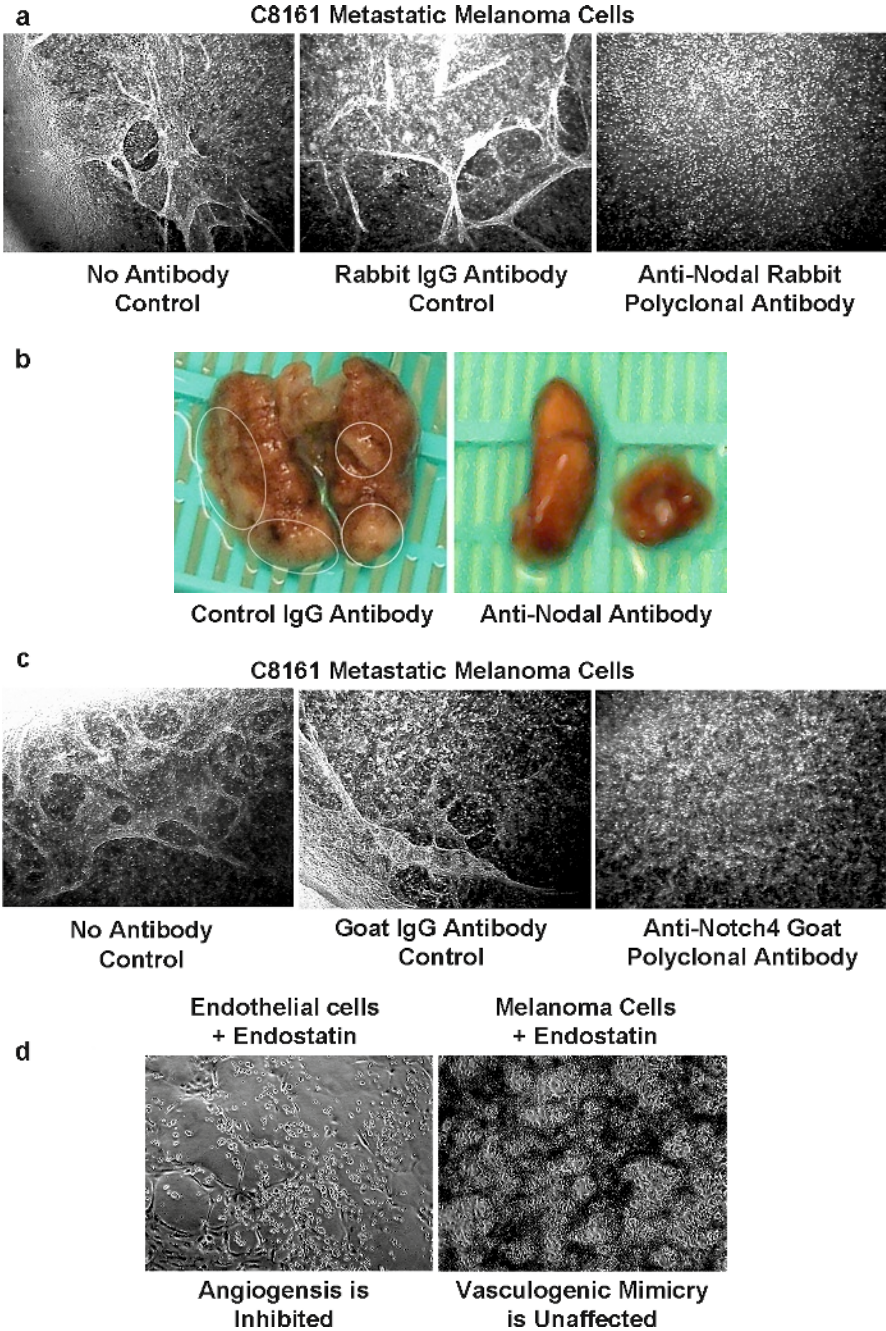


Fig. 13.5 Targeting the embryonic phenotype to suppress metastasis. **(a–b)**: Inhibition of Nodal activity with function-blocking antibodies blocks vasculogenic mimicry *in vitro* and lung colonization *in vivo*. **(a)** Metastatic melanoma cells (C8161) either untreated or treated with a control IgG antibody participated in vasculogenic mimicry on Type I collagen, whereas cells treated with anti-Nodal antibodies failed to form vascular-like networks. **(b)** Retro-orbitally injected C8161

cells in nude mice characteristically colonize to the lung (experimental metastasis). Injection of cells was followed with 10 days of intraperitoneal injections with control IgG or anti-Nodal antibodies, then animals were sacrificed and their lungs examined macroscopically. Lungs of control IgG-treated animals showed numerous metastases (*white circles*), while macroscopic evidence of tumor cell colonies was less apparent in lungs of anti-Nodal treated animals. (c) Notch4 receptor neutralizing antibodies inhibit vasculogenic mimicry *in vitro*. Untreated or control IgG-treated C8161 cells formed vascular-like networks in collagen I gel matrix, whereas cells treated with anti-Notch4 neutralizing antibodies failed to form vascular-like networks. Of note, cells treated with anti-Notch4 antibodies together with recombinant human Nodal protein display a recovery of vasculogenic mimicry (Hardy et al. 2010; data not shown), suggesting that Notch4 functions in Nodal-dependent vasculogenic mimicry. (d) The differential effects of Endostatin (an angiogenesis inhibitor) on endothelial cells compared with melanoma cells in 3-D gels of collagen I *in vitro*. Phase-contrast microscopy of human microvascular endothelial cells (*left*) and human aggressive melanoma cells (*right*) treated with Endostatin. Angiogenesis is inhibited in human microvascular endothelial cells, as shown by the lack of networks. Vasculogenic mimicry and network formation, however, are unaffected in the melanoma cells, as shown by the integrity of the morphologically distinctive, PAS-stained (*black*), vasculogenic networks (Original magnifications: (a) $\times 2$; (b) $\times 2$; (c) $\times 2$; (d) $\times 10$)

cross-talk between Nodal and Notch signaling pathways has provided new insights into the regulation of Nodal by Notch4, while independently showing the inhibitory effect of Notch antibody on melanoma vasculogenic mimicry (Strizzi et al. 2009b; Hardy et al. 2010) (Fig. 13.5). Collectively, the Nodal-Notch studies remind us of the importance of understanding the link between embryonic development and cancer, and may provide new insights for more effective therapies targeting the illusive embryonic phenotype of aggressive melanoma cells. Furthermore, ongoing studies focused on the reprogramming of melanoma cells by embryonic microenvironments are yielding new clues regarding the epigenetic reversion of the metastatic phenotype that might hold promising leads for adjuvant therapy.

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Abstract Melanoma represents an ideal experimental system to model the influence of tumor microenvironment on neoplastic cells given that a rich collection of cell types is localized in the physiological environment. Melanoma cells actively interact with the tumor microenvironment in a bidirectional manner by orchestrating the normal cells. Human epidermal melanocytes can readily be isolated and used as a paradigm to understand initiation and development of their malignant counterparts, melanoma cells. This chapter focuses on cell–cell communication between melanocytes and surrounding keratinocytes and fibroblasts, and summarizes key growth factors and inflammatory cytokines that are important for melanocyte function and homeostasis. We will then propose a model of malignant transformation of melanocytes, in which microenvironmental signals play key roles.

14.1 Introduction

Melanoma is the most deadly cancer in the skin with a 5-year survival rate of advanced, metastatic disease at 15%. Fortunately, recent years have witnessed breakthroughs in melanoma therapies such as targeted therapy, PLX4032 (RG7204) to block the MAPK signaling pathway by specifically targeting mutated oncogenic BRAF (Bollag et al. 2010; Flaherty et al. 2010), or immunotherapy by stimulating the immune response by blocking cytotoxic T-lymphocyte-associated antigen 4 using Ipilimumab (Hodi et al. 2010) (see also Chaps. 16 and 17).

Melanoma cells activate, recruit, and continuously interplay with other cells in the tumor microenvironment to facilitate their own growth, survival, migration, and invasion.

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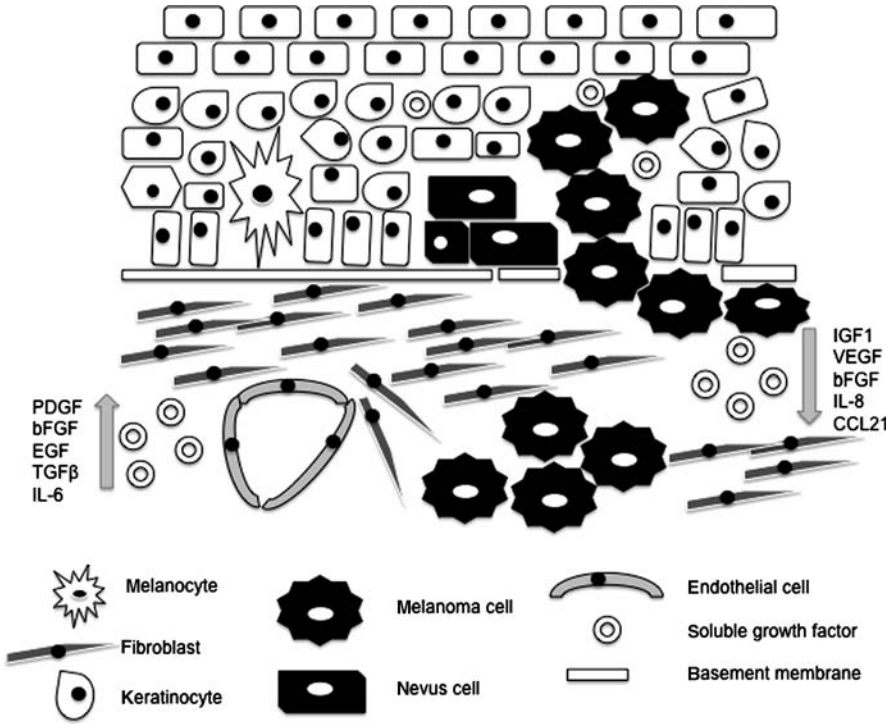


Fig. 14.1 Schematic representation of cross-talk between melanoma cells and tumor microenvironment. Normal melanocytes are localized at the basement membrane that separates epidermis from dermis and they are tightly regulated by surrounding keratinocytes through direct cell–cell contact. Upon transformation, the tight regulation mediated by E-cadherin is lost and replaced by N-cadherin between melanoma cells and adjacent cells. During melanoma development and progression, melanoma cells secrete soluble factors to recruit distant fibroblasts or immune cells in tumor microenvironment to its peritumoral zone. Stromal cells including fibroblasts, endothelial cells, and immune cells are activated and participate through paracrine signaling pathways to facilitate degradation of the basement membrane, invasion into the dermis, and metastatic dissemination. The dynamic communication between melanoma cells and the tumor microenvironment is mediated by direct cell–cell contact or soluble growth factors including bFGF, PDGF, VEGF, TGF β , cytokines, and inflammatory factors

As illustrated in Fig. 14.1, the tumor microenvironment of melanoma consists of neoplastic cells, normal cells, soluble growth factors, and extracellular matrix components that together cooperate to drive tumorigenesis. Perhaps the most striking evidence to support this notion came from our observation that metastatic melanoma cells lose their tumorigenic properties if forced to attach to keratinocytes (Hsu et al. 2000). Similarly, melanoma cells can be reprogrammed by an embryonic microenvironment to a benign phenotype (Díez-Torre et al. 2009; Gerschenson et al. 1986; Topczewska et al. 2006), further

underscoring the plasticity of malignant cells and the existence of inhibitory cues in the microenvironment.

14.2

Melanocyte, Melanoma, and the Microenvironment

Human melanocytes are specialized melanin-producing cells of neural crest cell origin. They are embedded at the conjunction of epidermis and dermis. All pigmented melanocytes are derived from embryonic neural crest cells that are highly migratory (White and Zon 2008). Multipotent neural crest stem cells (NCSC) can give rise to at least four differentiated cell lineages including melanocytes, glia, sensory neurons, and adrenal cells (Bronner-Fraser and Fraser 1988; Yu et al. 2006; Li et al. 2010). Several factors play a role in establishing multipotent neural crest stem cell, restricting neural crest stem cells toward a melanoblast fate, and driving differentiation of pigmented melanocytes (White and Zon 2008) (see also Chap. 13). Melanocytes make close contacts with keratinocytes, primarily through E-cadherin. On the basement membrane, the ratio between melanocytes and keratinocytes ranges from 1:5 to 1:8. Isolated melanocytes grow relatively rapidly in culture, and display bi- or tri-polar dendrites. In contrast, melanocytes rarely grow in the physiological setting of skin and display multiple (~35) dendrites to communicate with surrounding keratinocytes. It is the cell-cell contact through E-cadherins that closely regulates growth and proliferation of melanocytes and prevents early transformation of melanocytes to nevi (see also Chap. 2). With few exceptions, melanoma cells lose contact with keratinocytes and communicate with themselves and fibroblasts, which is due to the switch from E-cadherin to N-cadherin (Hsu et al. 2000; Li et al. 2001). Restoration of E-cadherin in melanoma cells of low levels of E-cadherin led to adhesion to keratinocytes, and inhibited invasion of melanoma cells into the dermis by downregulation of invasion-related molecules (Hsu et al. 2000).

There are several mechanisms for malignant transformation. Hypoxia is one of the significant characteristics of skin and tumor microenvironment and can accelerate transformation (Bedogni et al. 2005; Monsel et al. 2010). These experiments have provided an important hint that the surrounding tissue may contribute to transformation. Combination of bFGF and ultraviolet (UV) B could readily lead to pigmented lesions, some of which resemble low-grade melanoma (Berking et al. 2001). Interestingly, melanocytes display melanoma-like aggressive phenotypes when grown on melanoma cell-derived matrix (Seftor et al. 2005), suggesting that in addition to genetic approaches, the microenvironment can also reprogram normal melanocytes into melanoma-like phenotypes. Conversely, malignant cells can be reprogrammed by human or chick embryonic stem cell matrix to a benign melanocyte-like phenotype (Kulesa et al. 2006; Postovit et al. 2006) (see also Chap. 13). Together, these examples demonstrate how the surrounding microenvironment can play a role in modulating many aspects of both normal and malignant melanocytes.

14.3
Growth Regulation of Human Melanocytes by Epidermal
Keratinocytes and Dermal Fibroblasts

Human epidermal melanocytes isolated from fetal or adult skin do not propagate *in vitro* unless cultured in a defined growth medium including phorbol ester and cholera toxin (Eisinger and Marko 1982) or growth factors that replace their activities. Condition medium derived from melanoma or astrocytoma cells support the growth of melanocytes in the absence of growth factors suggesting that malignant cells produce their own growth factors for autocrine stimulation (Table 14.1 and Eisinger et al. 1985).

Melanocytes are closely surrounded in skin by keratinocytes and survive and proliferate when co-cultured with keratinocytes or in the presence of keratinocytes-conditioned medium, suggesting that most melanocyte growth-stimulating factors are derived from keratinocytes (see also Chap. 2). Basic fibroblast growth factor (bFGF) is produced by keratinocytes to enhance melanocyte proliferation (Halaban et al. 1988). Not only does bFGF have an impact on melanocyte survival, growth, and proliferation, bFGF can also promote melanocyte migration via phosphorylation of focal adhesion kinase (p125^{FAK}) (Wu et al. 2006). α -MSH stimulates melanocyte growth by binding to its high affinity receptor and its activity requires bFGF and/or activation of protein kinase C (De Luca et al. 1993). Upon exposure of human keratinocytes to UVB, secreted interleukin 1- α and β (IL1- α and β) from keratinocytes stimulate the secretion of endothelin (ET-1), which plays an important role in melanocyte proliferation (Imokawa et al. 1992; Jamal and Schneider 2002). Similarly, UVA/B-irradiated keratinocytes secrete granulocyte/macrophage colony-stimulating factor (GM-CSF), which also stimulates DNA synthesis and differentiation of melanocytes in a dose-dependent manner (Hirobe et al. 2004; Imokawa et al. 1996).

There is no apparently direct contact between epidermal melanocytes and dermal fibroblasts in skin. However, fibroblasts can produce growth factors that are important for melanocytes including bFGF and hepatocyte growth factor (HGF). IL1- α from UVB-irradiated

Table 14.1 Growth factors or inhibitors for melanocytes derived from human epidermal keratinocytes and dermal fibroblasts

Melanocyte growth regulator	Keratinocytes	Fibroblasts
α -MSH	+++	NA
bFGF	+++	++
ET-1	+++	NA
ET-3	NA	+++
HGF	NA	+++
GM-CSF	+++	++
SCF	+++	+
DKK1	NA	+++

keratinocytes can stimulate the production of HGF in fibroblasts (Mildner et al. 2007). Interestingly, HGF also plays a role in promoting melanocyte motility depending on CD44v6 expression (Damm et al. 2010), and protecting melanocytes from apoptosis in a MITF-dependent manner (Beuret et al. 2007). Another paracrine factor, neuregulin-1, is derived from dermal fibroblasts. It can effectively increase pigmentation of melanocytes in monolayer and the reconstructed skin model (Choi et al. 2010). On the other hand, dermal fibroblasts can have an inhibitory effect on density, proliferation, and differentiation of melanocytes via secreted DKK1 (Yamaguchi et al. 2007). DKK1 is highly expressed by fibroblasts and can suppress β -catenin and MITF in melanocytes, which are essential for melanocyte growth and proliferation. Treatment of keratinocytes with DKK1 can increase their proliferation and decrease their uptake of melanin (Yamaguchi et al. 2008).

14.4

Inflammatory Mediators on Human Melanocyte Function

The epidermal and dermal microenvironment harbors inflammatory mediators, cytokines, hormones, and growth factors that can alter human melanocyte function in a profound way. Many of these factors are released into the microenvironment after UV exposure, and subsequently affect melanocyte pigmentation, proliferation, differentiation, cytokine production, and motility.

Tumor necrosis factor- α (TNF- α) is among many cytokines responsible for an inflammatory response in skin. Upon UVB irradiation, keratinocytes release TNF- α , which is likely stimulated by IL-1 α . IL-1 α is the primary mediator that responds to inflammation and injury in skin. Secretion of IL-1 α increases as keratinocytes and fibroblasts age (Okazaki et al. 2005). It has been reported that increased secretion of IL-1 α can stimulate production of HGF by dermal fibroblasts in a paracrine manner, and production of ET-1 by keratinocytes for autocrine stimulation, which together stimulate proliferation of melanocytes and induce their tyrosinase activity. Nuclear receptor retinoid X receptor- α (RXR- α) expressed in keratinocytes is one of the upstream regulators of these cytokines and can protect keratinocytes and melanocytes from UV-induced DNA damage and enhance proliferation via modulation of secretion of heparin-binding EGF-like growth factor, GM-CSF, IL-1 α , and cyclooxygenase-2 and activation of mitogen-activated protein kinase pathways (Wang et al. 2011). Depletion of RXR- α in epidermal keratinocytes leads to alterations in expression of keratinocyte-derived secreted factors such as ET-1, SCF, HGF, FGF2, and α -MSH, which modulate proliferation and activation of melanocytes after UV irradiation (Wang et al. 2011).

Melanocytes express IL-8 mRNA when stimulated with TNF- α or IL-1 α , and the supernatants from stimulated melanocyte cultures become positive for neutrophil and monocyte chemotactic activity, suggesting a possible role of melanocytes in participating in the initiation of an inflammatory response (Zachariae et al. 1991). Treatment of melanocytes with TNF- α , IL-1 α , or IL-6 leads to inhibition of tyrosinase activity and DNA synthesis (Swope et al. 1991), suggesting that they function as paracrine factors. Since melanocytes synthesize and transfer melanin to keratinocytes upon UV irradiation, it may implicate a negative feedback loop consisting of these factors to modulate melanocyte

function. α -MSH can serve as a primary anti-inflammatory factor by opposing TNF- α -induced NF- κ B activity in human melanocytes (Haycock et al. 1999). It is mainly produced by epidermal keratinocytes suggesting intricate interactions between keratinocytes, melanocytes, and inflammatory mediators.

14.5

Interplay Between Inflammation and Tumor Initiation, Promotion, and Progression

Ample evidence has pointed to a role of chronic inflammation in underlying tumor initiation and development (Grivennikov et al. 2010; Luo et al. 2004). IL-6/STAT3 and IKK β -dependent NF- κ B signaling pathways are key players linking inflammation and cancer by regulating an array of cytokines (He and Karin 2010; Yu et al. 2009). Inflammation contributes to onset of malignancies by several potential mechanisms including, 1. stimulated cell proliferation can increase the likelihood of acquiring the transforming mutation, 2. production of metabolites such as reactive oxygen species (ROS) or endothelial nitric oxide synthase (eNOS) can cause DNA damage, 3. suppression of cell-mediated immune response can create an environment that enhances tumor growth, and 4. inhibition of apoptosis (Kitasato et al. 2007; Rubin et al. 2004). An immunohistochemistry study demonstrated that chronic inflammation could contribute to malignant transformation in the human upper airways by production of eNOS (Pacova et al. 2009).

UVB is regarded as an environmental carcinogen that is critical for melanoma development. UVB but not UVA can initiate melanoma using the HGF mouse genetic model (De Fabo et al. 2004). Exposure of keratinocytes to UV increases ROS production (Yoshihisa et al. 2010). In murine fibroblasts, UV irradiation enhanced ROS production via PKC δ signaling (Bossi et al. 2008). Human epidermal keratinocytes have higher basal hydrogen peroxide (H₂O₂) levels than melanocytes, and can transfer hydrogen peroxide to melanocytes (Pelle et al. 2005). UVB and UVC can differentially activate STAT3 in human keratinocytes and fibroblasts via ROS and DNA damage (Bito et al. 2010). Together, these results suggest that human epidermal keratinocytes and/or dermal fibroblasts represent major sources of ROS for epidermal melanocytes following UV irradiation and initiate inflammatory response via the STAT3 signaling pathway.

It has been noted that UVB, along with growth factor such as bFGF, can transform normal melanocytes (Berking et al. 2001, 2004). However, the underlying molecular basis of malignant transformation remains unclear. Given the evidence that ROS and DNA damage are implicated in UV irradiation-induced inflammatory response in skin keratinocytes and fibroblasts, an intriguing possibility is that UV-mediated inflammatory responses in the skin environment, including generation of ROS and release of secreted cytokines, could further cause DNA damage in epidermal melanocytes, and contribute to genetic alterations. To experimentally test this, we are currently using a skin reconstruct model involving co-culture of epidermal keratinocytes, melanocytes, and dermal fibroblasts, to investigate the role of UVB irradiation in malignant transformation of melanocytes via ROS production by inflammatory cells and DNA damage by both UV and inflammatory mediators (Fig. 14.2).

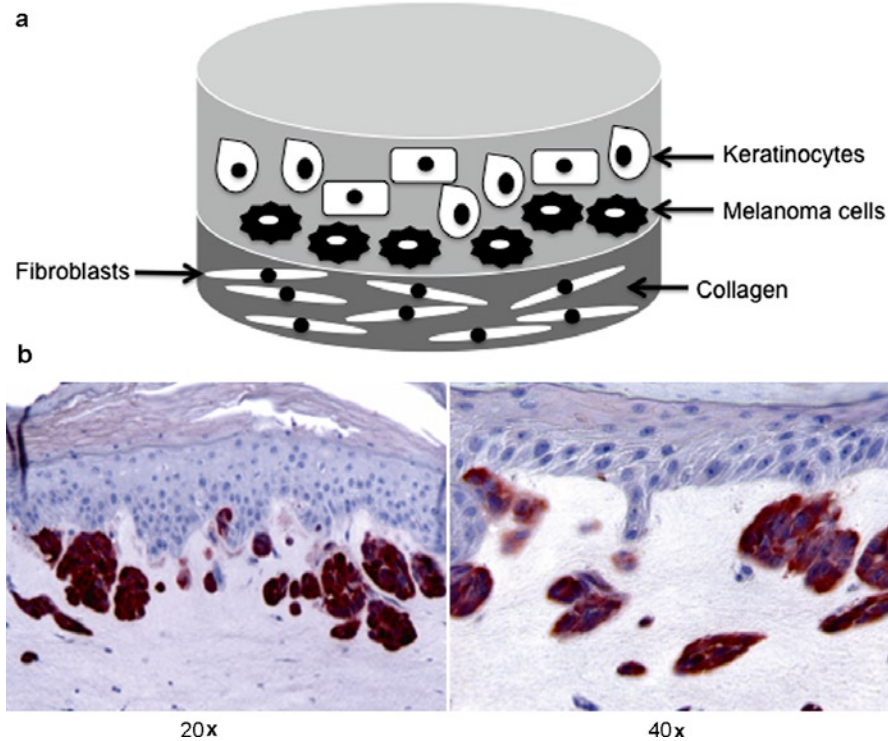


Fig. 14.2 Human skin reconstruct using the 3-D organotypic culture system. **(a)** A schematic representation of human skin reconstruct at which human melanocytes or melanoma cells are grown as 3-D with keratinocytes, fibroblasts, and collagen that mimic the microenvironment. **(b)** VGP human melanoma cells WM3248 are grown in 3-D skin reconstruct comprising of keratinocytes, fibroblasts, and collagen. Typical invasion into dermis of WM3248 cells is a characteristic of RGP melanoma line. Images are captured at 20 \times and 40 \times (Courtesy of Dr. Ling Li, the Wistar Institute)

14.6 Conclusion

Transformation of normal melanocytes can be partially attributed to dysregulated tissue homeostasis executed by keratinocytes. Furthermore, transformed melanocytic cells send signals throughout the microenvironment to recruit other cells types. Those cells become activated, communicate with the neoplastic cells, and elicit soluble pro-survival and anti-apoptosis signals through autocrine or paracrine signaling patterns or direct cell–cell contacts in order to create a suitable tissue microenvironment that supports melanoma progression. Over the decades, experimental studies have identified key intrinsic signaling pathways that mediate transformation of melanocytes into melanoma cells. In the skin, melanocytes are mainly surrounded by epidermal keratinocytes and they also communicate

with dermal fibroblasts. How the microenvironment of melanocytes plays a role in transformation remains to be clarified. We propose to use human melanocytes as a paradigm to study and understand cell–cell communication and growth regulation between melanoma and the microenvironment. An understudied area is the role of inflammation in melanoma development and progression. We hypothesize that UVB irradiation induces inflammatory responses through generation of ROS and DNA damage. Subsequently, these events lead to release of an array of cytokines, and inflammatory mediators. ROS can also be transferred to melanocytes, and in combination with inflammatory cytokines, likely causes DNA damage in melanocytes.

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Abstract The majority of basic research until now is still performed using two-dimensional (2-D) cell culture studies with tumor cell lines grown in monolayer as models for tissues. However, cancer biologists increasingly recognize that tumor cells kept in monolayer culture poorly represent the behavior of tumors *in vivo*. Importantly, they do not adequately recapitulate the tumor microenvironment which critically determines tumor cell proliferation, invasive growth, and metastatic spread. More sophisticated model systems are required not only to better understand the biological basis of these processes but also to more effectively evaluate anticancer drug candidates. In [Sect. 15.1](#) we will review the currently available complex *in vitro* cell culture model systems which are used in melanoma research. In [Sect. 15.2](#) we will describe the spectrum of experimental mouse models that have been developed over the past decades and discuss some of the relevant strengths and weaknesses of the individual approaches.

Keywords Melanoma • Complex model systems • Organotypic skin culture • Spheroid • Transgenic mice

15.1

Complex *In Vitro* Cell Culture Model Systems

15.1.1

Introduction

Although there is experimental evidence that human tumor cell lines grown in culture can be representative of the original tumor lesion (Masters 2000; Smalley et al. 2006b; Meier et al. 2000), it is obvious, that tumor cells *in vivo* grow in an environment consisting of extracellular matrix components, stromal cells, inflammatory cells, and endothelial cells (Bissell and Radisky 2001). The contribution of the tumor microenvironment to tumor

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progression has already been recognized in 1889 by the “seed and soil” theory of Paget (Paget 1889) and later extended and confirmed by Meenhard Herlyn's group (Li et al. 2003). This theory claims that not only genetic changes in the tumor cells determine an aggressive phenotype, but that microenvironmental factors have an impact on tumor cell behavior. Factors secreted by stromal cells as fibroblasts or direct cell–cell contacts between the tumor cells and the surrounding stromal cells may either inhibit malignant transformation or promote tumor progression (Li et al. 2003). Cells grown in monolayer can differ considerably in their morphology, cell–cell and cell–matrix interactions, and differentiation from those growing in more physiological three-dimensional (3-D) environments (Yamada and Cukierman 2007). Furthermore, 3-D culture gene expression profiles have been shown to more accurately reflect clinical expression profiles than those observed in 2-D cultures. Finally, preclinical drug screening in 3-D culture models more reliably predict clinical efficacies, and monolayer culture of tumor cells has remained a poor predictor of a patient's response toward therapeutic agents (Johnson et al. 2001; Voskoglou-Nomikos et al. 2003; Burdett et al. 2010) (Table 15.1).

Animal models are not suitable for high throughput screening, but often provide definitive tests of the importance of specific molecules or drug targets. However, one has to keep in mind that animal models may not adequately reproduce features of human tumors or therapeutic responses (Kung 2007; Teicher 2006; Burdett et al. 2010). On the other hand, most 3-D *in vitro* models lack the complex vascular systems that perfuse tissues *in vivo*. Since “Life isn't flat” (Smalley et al. 2006b), *in vitro* 3-D tissue models provide an approach to bridge the gap between traditional cell culture and animal models (Griffith and Swartz 2006; Rangarajan et al. 2004; Yamada and Cukierman 2007).

15.1.2

Culture of Melanoma Cells in Extracellular Matrix Scaffolds

The specific extracellular matrix microenvironment provided to cells can substantially influence experimental outcome. Both, the composition and stiffness of the extracellular matrix surrounding the cells have major effects on signaling and behavior (Yamada and Cukierman 2007). Therefore, a widely used strategy is to propagate cells in tissue culture and then implant them in a 3-D extracellular matrix scaffold as either single cells or as tissue-like aggregates. Three-dimensional scaffolds have been generated from purified molecules such as collagen I, matrigel, or from native extracellular matrices secreted and isolated from fibroblasts or keratinocytes.

Because of its ubiquitous nature and relative ease of isolation, collagen was one of the earliest biomaterials to be widely used for 3-D cell culture. Collagen gel embedding involved the encapsulation of small tumor explants (1–2 mm) or of dissociated tumor cells within a collagen matrix that allowed the explants to maintain their viability and cellular architecture *ex vivo* (Burdett et al. 2010). Cells suspended in extracellular matrix components are easily visualized by phase contrast or immunofluorescence microscopy. In addition, the biological response of cells suspended in this 3-D system can be compared to that of cells grown in monolayer. Viable cells may also be removed from the 3-D cell culture system for further experimentation, including biological analysis and flow

Table 15.1 Complex *in vitro* cell culture model systems

Melanoma model systems	Application	Advantages	Disadvantages
Culture in extracellular matrix scaffolds	Influence of extracellular matrix components on melanoma cell growth, invasion and drug sensitivity	Easy to perform; different matrix components can be tested	Extracellular matrix components absorb drugs or growth factors; mostly higher drug concentrations have to be used
Microcarrier bead culture	High cell density culturing in matrix components possible	Several carrier materials can be used; ideal for large-scale culturing in matrix components	Expensive and more complicated
Transwell chambers	Analysis of chemotaxis, migration, invasion	Easy to perform when precoated transwells are used, fast assay	Expensive, high variations possible if individual coating of the inserts is performed
Organotypic explant culture	Invasion, migration	Culturing of melanoma cells in a physiological environment	Limited culture time; patient material necessary
De-epidermized dermis (DED)	Influence of extracellular matrix components and basal membrane on melanoma cell invasion	Native extracellular matrix and basal membrane present	Limited culture time; patient material necessary
2-D coculture systems	Influence of growth factors or cell–cell contact on melanoma cell growth, survival, invasion, gene expression	Easy to perform, secreted factors as well as cell contact can be analyzed separately	Patient material necessary for primary cells; sometimes separation of the cells necessary before analysis
Mono- or multi-cellular spheroids	Influence of melanoma cells grown in aggregates on drug sensitivity, secreted factors or cell–cell contact with melanoma cells itself or other cell types	Easy to perform, fast assay, high throughput screening possible, variable platforms and conditions possible	Suboptimal physiological conditions; limited informative value about melanoma invasiveness, not all melanoma cells can be used
Organotypic skin equivalent culture	Growth, survival, migration, and invasion of melanoma cells in a physiological environment	Most sophisticated melanoma model system, physiological environment, dermal and epidermal culture systems available	Laborious, Expertise needed, not all melanoma cells can be used

cytometry. Collagen gels can mimic loose or dense connective tissue depending on the concentration of collagen; such gels have been used widely in studies of fibroblast and tumor cell migration and signaling (Grinnell 2003). Since each tissue *in vivo* has a characteristic matrix microenvironment, for a given study it is crucial to select an appropriately matched 3-D *in vitro* matrix (Yamada and Cukierman 2007). Besides collagen, the basal membrane components fibronectin, laminin, and collagen IV as well as matrigel (BD Bioscience, SanJose, CA) have been used for melanoma cell culture. Matrigel is a basement membrane extract derived from the Engelbreth-Holm-Swarm mouse sarcoma that contains a diverse array of components, including collagen type IV, laminin, and other ECM molecules, as well as various soluble factors, such as cytokines and growth factors (Burdett et al. 2010). However, because matrigel is a largely undefined and variable mixture of proteins, Matrigel culture has not been widely used for drug screening purposes.

More sophisticated models use other biomaterial scaffolds for culturing melanoma cells on other natural substrates (Fischbach et al. 2007; Zhang et al. 2010). Prototypical scaffolds were developed, in which freeze-dried and then cross-linked solutions of collagen and glycosaminoglycans create biodegradable, sponge-like structures (Griffith and Swartz, 2006). Hyaluronic acid can also be used as a scaffold for bioengineered skin (Scuderi et al. 2008). Furthermore, native extracellular matrix components secreted by fibroblasts can be isolated by a protocol described by Beacham (Beacham et al. 2007). The advantage of using native extracellular matrix components is that the natural composition and 3-D structure of the matrix molecules remain intact. Extracellular matrix components can serve as a reservoir for growth factors secreted by neighboring cells or melanoma cells and by this can influence morphology and growth of neighboring cells. Together with fibroblasts, immune cells, and blood vessels the extracellular matrix builds the tumor stroma and influences tumor progression, invasion, and metastasis (Mueller and Fusenig 2004).

15.1.3

Microcarrier Bead Culture

Microcarrier beads are also widely used (Smit et al. 1995). A microcarrier is a support matrix allowing for the growth of adherent cells in bioreactors. Carrier materials for cells may be composed by gelatin, porous glass, collagen, or cellulose, with dimensions of 170–6,000 μm . In microcarrier culture, cells grow as monolayers on the surface of small spheres or as multilayers in the pores of macroporous structures that are usually suspended in culture medium by gentle stirring. By using microcarriers in simple suspension culture, fluidized or packed bed systems, yields of up to 200 million cells per milliliter are possible. Therefore, microcarriers provide convenient surfaces for growing animal cells or increasing the yield of cells from standard monolayer culture vessels and perfusion chambers. The high cell density confers more stability and improves the longevity of the culture, making macroporous microcarriers suitable for long-term culture. It has been described that normal human melanocytes or melanoma cells can be coated on microcarrier beads and polymerized within fibrin or fibrin-collagen hydrogels. A layer of dermal fibroblasts was added to more accurately simulate the microenvironment. Invasion can be monitored over

a time period of 7 days. This model shows that the melanoma cell lines recapitulate their *in situ* growth patterns in this environment (Ghajar et al. 2007).

Microcarrier beads can also be rolled on a lymph node endothelial surface, which was created by growing endothelial cells on a differentiating extract of lymph node bioma-trix, and testing the ability of tumor cells to invade across matrigel-coated filters. Interestingly, compared to the contact with plastic, Lewis lung carcinoma and B16 melanoma cell invasiveness was increased after exposure to “lung endothelial surface.” This indicates that a lymph node environment may modulate the metastatic potential of tumor cells (Whalen et al. 1994).

15.1.4

Transwell Chambers

Transwell chambers are often used to study migration or invasion of tumor cells. This system is also called Boyden chamber assay, originally introduced by Boyden for the analysis of leukocyte chemotaxis (Boyden 1962). It is based on a cylindrical cell culture insert nested inside the well of a cell culture plate. It consists of chambers of two medium-filled compartments separated by a microporous membrane. The insert contains a polycarbonate membrane at the bottom with a defined pore size. Depending on the composition of the porous membrane, either migration or invasion of tumor cells can be studied. Usually, cells are seeded in the upper compartment in serum-free media, while serum or similar chemoattractants are placed in the well below. Migratory cells move through the pores toward the chemoattractant below. The number of cells that have migrated to the lower side of the membrane is determined after staining the membrane and quantified in a plate reader. Therefore, the Boyden chamber-based cell migration assay has also been called filter membrane migration assay, transwell migration assay, or chemotaxis assay. A number of different Boyden chamber devices are commercially available (Chen 2005). Such a system can be used to obtain an objective numerical readout to assess the effects of drugs or the modulation of target gene expression on cell migration. Furthermore, by the addition of a matrigel layer on top of the membrane, this assay can be modified to measure invasion, such that cells have to invade through the matrix to reach the underside of the filter.

15.1.5

Organotypic Explant Culture: Ex Vivo Cultures

Organ explant slices can be cultured on a semiporous membrane or are embedded in a 3-D collagen gel (Pampaloni et al. 2007). Organotypic slice cultures preserve the cyto-architecture and cellular differentiation of the original tissue. Although this approach benefits, in that epithelial/endothelial cells are cultured in a relatively physiologically normal microenvironment, the culture period during which the organ remains viable is limited. A problem is also how to obtain the starting material, especially if it is of human origin (Hegerfeldt et al. 2002; Berry et al. 1975; Friedl et al. 2004). With this method one can perform 3-D invasion assays which support the invasion of tumor cell clusters from cancer explants.

15.1.6

De-Epidermized Dermis (DED)

The skin model based on de-epidermized human dermis populated with keratinocytes and fibroblasts was originally developed for resurfacing burned patients (Ghosh et al. 1997; Chakrabarty et al. 1999; Sahota et al. 2003; Harrison et al. 2006). On de-epidermized dermis (DED) stromal cells, keratinocytes, or melanoma cells can be seeded and cultured for several days and the invasive capacity of melanoma cells in this environment can be analyzed (Dekker et al. 2000). Using this model one can evaluate the contribution of the extracellular matrix, the basal membrane or normal skin cells on melanoma cell invasion. The advantage of this system is that the de-epidermized dermis retains a native extracellular matrix and basal membrane and the contribution of the basal membrane in the invasive properties of melanoma cells can be analyzed easily. This *in vitro* system showed reliable invasion of highly invasive cells and was used to investigate the proteolytic mechanisms involved in melanoma cell invasion into dermal connective tissue (Dennhofer et al. 2003). By culturing melanoma cells either on the dermal side of the DED or on the basal membrane side one can study the influence of the basement membrane on the invasive behavior of melanoma cells (Van Kilsdonk et al. 2010). It is also possible to study the role of matrix metalloproteases in this model on melanoma cell invasion (Dennhofer et al. 2003). The basement membrane components can also be removed by treatment of the DED by incubation with dispase (Harrison et al. 2006). Using this model it has been shown that the dermoepidermal basement membrane can prevent invasion of metastatic melanoma cell lines in the absence of a stratified epidermis (Van Kilsdonk et al. 2010).

15.1.7

2-D Coculture Systems

The 2-D coculture system is ideally suited to analyze the effect of defined factors or genes especially on melanocyte transformation in an environment in which at least a quasi *in vivo* like cellular communication can take place. It is known that melanocytes cultured *in vitro* display different phenotypic characteristics than melanocytes *in vivo*. This suggests a role of microenvironmental signals in controlling the melanocytic phenotype. Indeed, it was shown that upon coculture with undifferentiated keratinocytes melanocytes regain their normal phenotype resembling those *in situ* indicating that keratinocytes regulate cell growth, dendricity, and antigen expression of melanocytes *in vitro* (Hsu et al. 2002; Shih et al. 1994). Following malignant transformation, the dominance of keratinocytes over the melanocytic phenotype is lost and melanoma cells become more and more autonomous (Valyi-Nagy et al. 1993; Shih et al., 1994; Hsu et al. 2000, 2002). To analyze independently the effects of soluble factors, the coculture can be performed in Transwell systems where melanocytes and keratinocytes are seeded in two compartments of a tissue culture well, which is separated by a semiporous membrane. By this it was shown that E-cadherin-mediated cell adhesion is required for keratinocyte-mediated control of melanocytic cells (Li et al. 2004).

Cultivation of melanoma cells with normal dermal fibroblasts indicates that melanoma cells can direct gene expression in fibroblasts (Gallagher et al. 2005; Loffek et al. 2005;

Ntayi et al. 2003). Interestingly, highly aggressive melanoma cells cannot only direct the activation and functional differentiation of stromal fibroblasts and endothelial cells but also can transdifferentiate by masquerading as endothelial cells to take over all or part of stromal functions, a phenomenon termed as vasculogenic mimicry (Maniotis et al. 1999; Hendrix et al. 2003, 2007).

15.1.8

Mono- or Multicellular Spheroids

Spheroids, or tumor cell aggregates, have been used since the 1970s, mostly for investigations into the mechanisms of action of radiotherapy and chemotherapeutic drugs as well as drug resistance (Hirschhaeuser et al. 2010; Mueller-Klieser 1997; Smalley et al. 2006b). The principle is that melanoma cells are grown under non-adherent conditions, which permits the formation of 3-D aggregations or spheroids. Once formed, the spheroids are implanted into a matrix of collagen I, which mimics the microenvironment of human skin.

Cellular spheroids take advantage of many cell types to aggregate. The cellular aggregates can range in size from 20 to 1 μm in diameter, depending on the cell type and growth conditions. However, fewer than 100 human tumor cell lines have been shown to have the capacity to grow in spheroid cultures (Friedrich et al. 2007, 2009). Melanoma cells are more suitable for spheroid formation since it was shown that 18 of the 26 analyzed melanoma cell lines can form spheroids (Smalley et al. 2006a, b). Spheroids more closely resemble the *in vivo* situation than monolayer conditions due to the architecture and the extensive cell–cell contacts provided by the spheroids. Spheroids exhibit many of the biological properties of solid tumors including cell morphology, growth kinetics, gene expression, and drug response (Friedrich et al. 2007, 2009; Hirschhaeuser et al. 2010; Kunz-Schughart et al. 2004; Mueller-Klieser 1997). Human tumor spheroids are widely used for drug screening since cancer cells grown in these spheroids show greater resistance toward several anticancer drugs compared to cells grown in monolayer (Smalley et al. 2006b). This may be explained in part by increased cell–cell contact, 3-D cellular architecture, enhanced deposition of tumor-derived ECM within the spheroid, a lower overall cell proliferation rate, or a combination of these factors (Bates et al. 2000; Hamilton 1998; Burdett et al. 2010). Interestingly, similar to the situation in a tumor, a diffusion gradient exists within spheroids for oxygen and nutrients limiting the availability of these compounds to the innermost cells (Lin and Chang 2008).

In spheroids, melanoma cells form concentric, spherical structures that contain large proliferating cells in the periphery and smaller quiescent cells on the interior of the sphere (Fig. 15.1a, b). These spheroids can be implanted into a collagen-based matrix where they exhibit an invasive phenotype that is indicative of the progression stage of melanoma (Smalley et al. 2006b). This system can be further explored by interspersing fibroblasts into the collagen before imbedding into a spheroid. These fibroblasts infiltrate the spheroid and produce ECM proteins (Smalley et al. 2005) (Fig. 15.1a, b). Spheroids can be established from a single cell type or can be multicellular mixtures of tumor, stromal, and immune cells (Hirschhaeuser et al. 2010). Several cell types such as fibroblasts and immune

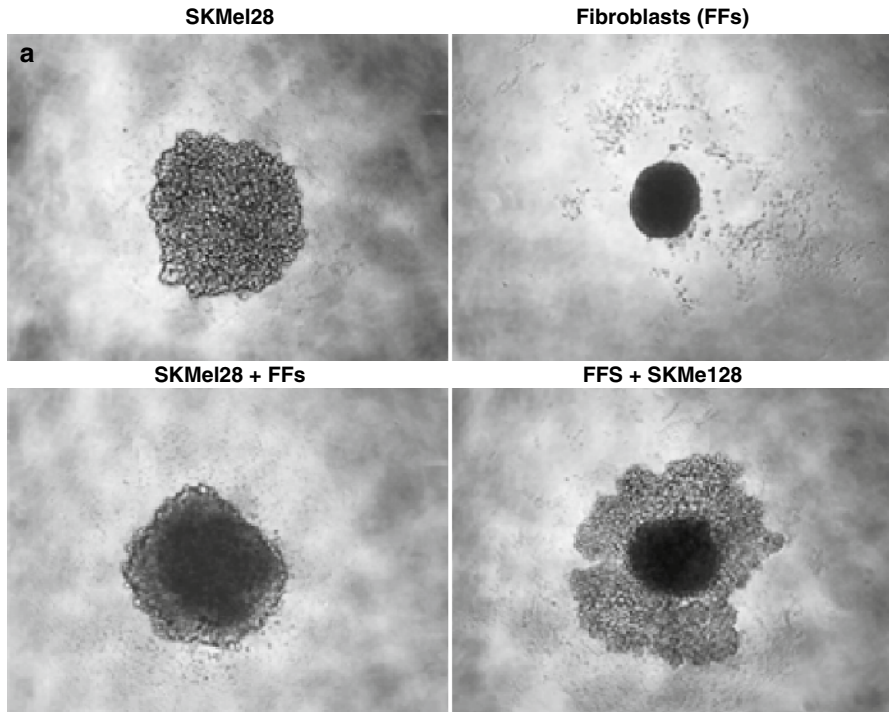


Fig. 15.1 Spheroid and organotypic skin equivalent models **(a)** Phase contrast pictures of spheroids of SKMel28 melanoma cells or primary dermal fibroblasts (FFs) 48 h after aggregation. Lower left picture: Spheroids of SKMel28 melanoma cells were formed for 24 h and afterward cocultivated for 24 h with dissociated fibroblasts for further 24 h. Lower right picture: Spheroids of primary fibroblasts were formed for 24 h and afterward cocultivated for 24 h with SKMel28 melanoma cells for further 24 h. **(b)** Melanoma spheroids either untreated (control) or treated with a beta-catenin inhibitor (Sinnberg et al. 2010). Spheroids were stained with the vitality stains calcein-AM and ethidium bromide to visualize live and dead cells, respectively. As a measure of cell death induction ethidium bromide fluorescence indicates dead cells. **(c)** Organotypic epidermal skin equivalents (SE). Shown are hematoxylin/eosin stainings of organotypic epidermal skin reconstructs with either integrated primary melanocytes (marked with an arrow), a radial growth phase (RGP), vertical growth phase (VGP), or metastatic melanoma cell line. We have observed that skin reconstructs consisting of human fibroblasts and keratinocytes simulate human skin *in vivo* and that human melanocytes or melanoma cells from different stages of melanoma development and progression recapitulate in skin reconstructs the biological behavior *in vivo*. In the skin reconstructs, only an irregular basement membrane is formed as seen by the collagen IV staining (pictures taken with permission from (Meier et al. 2000))

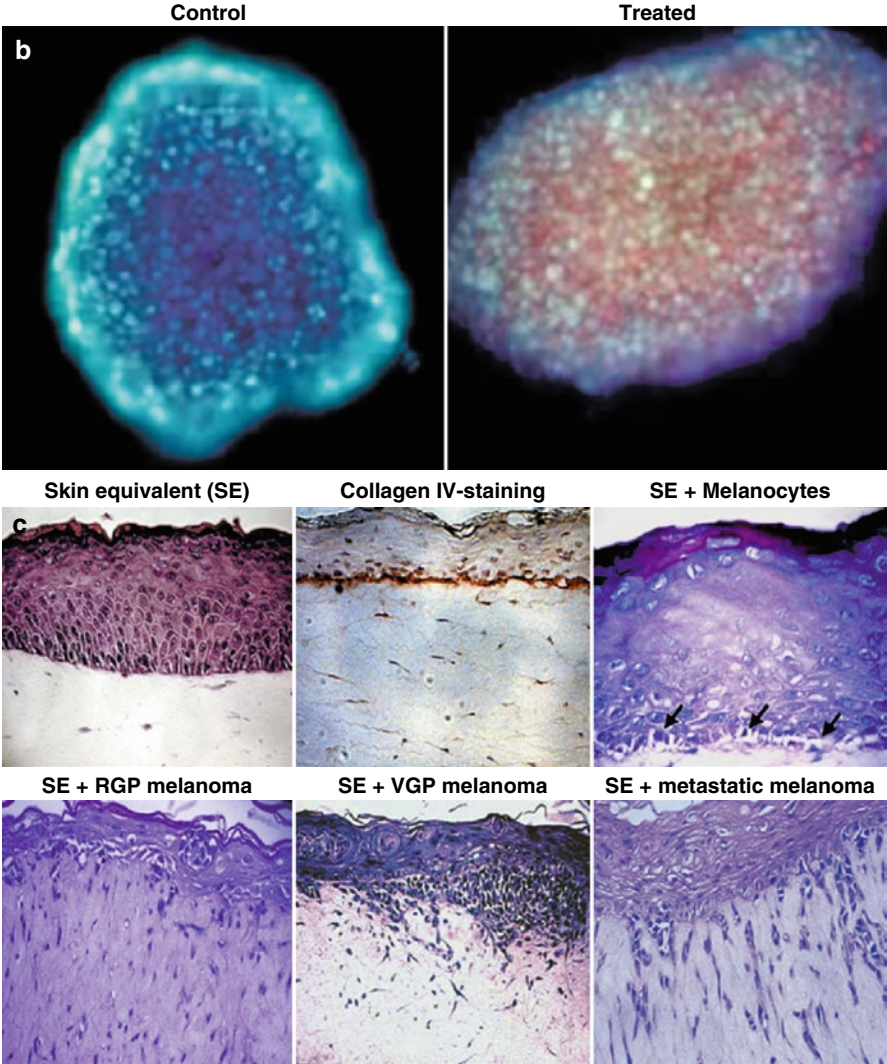


Fig. 15.1 (continued)

cells have been described, which can be successfully mixed with tumor spheroids under coculture conditions (Smalley et al. 2006b). Using this model system it was also shown recently that zebrafish embryo extracts promote sphere-forming ability of human melanoma cell lines (Na et al. 2009). Preformed multicellular tumor spheroids can be incubated with immune cells that migrate into the spheroid. As a result, tumor-associated migration and differentiation processes as well as cytotoxic and cytostatic activity of migrated immune cell populations can be examined (Konur et al. 1996; Gottfried et al. 2006; Pampaloni et al. 2007).

Spheroid formation can be induced by a variety of different techniques. In the spinner flask culture, fluid turbulence prevents attachment and promotes cellular aggregation (Sutherland et al. 1971). In the rotary wall vessel, reactor cells are placed between rotating cylindrical walls to mimic microgravity (Lin and Chang 2008; Friedrich et al. 2007). A simpler method, which does not need specialized equipment or additional processing, is the liquid overlay method. This involves the liquid overlay of a cell suspension over a non-adherent surface, such as agar-coated plates (Yugas et al. 1977; Burdett et al. 2010). The absence of fluid flow using this method results in a more pronounced diffusional gradient within the spheroids. Additionally, the spheroids formed through liquid overlay are more varied in size and number. To optimize this and to get more consistent spheroid size and composition, recent adaptations involve spheroid formation within hanging drops (Kelm et al. 2003; Timmins and Nielsen 2007) and microfluidic chips (Wu et al. 2008; Burdett et al. 2010). However, these methods need again specialized equipment and additional handling steps.

In contrast, the collagen-implanted spheroid model is easier and faster to perform and is suited to high throughput drug screening studies. This assay takes account of both cell–cell contact between adjacent tumor cells and the need for 3-D supporting matrix. It is based on the liquid overlay method in which a uniform cell suspension is plated on top of hard (1.5%) agar, which prevents the tumor cells from adhering to the underlying tissue culture plastic. After 48–72 h, the tumor cells form small aggregates, or spheroids. These spheroids can be used directly for drug toxicity studies. The protocol developed by Smalley et al. (2006a) differs in that the spheroids are harvested and mixed with a suspension of bovine collagen type I. These are then plated on a 24-well plate, which is already layered with collagen to prevent the spheroids from settling onto the underlying plastic. These collagen-implanted spheroids can be used for the analysis of the invasive capability of melanoma cells. It was shown that the extent of collagen invasion correlated with the tumor stage and the cells from the early stages were poorly invasive, whereas the cells from the metastasis colonized the entire collagen gel (Smalley et al. 2006a, b). The spheroids are scored for cell survival by removing the cell culture media, washing in PBS, and then staining using calcein-AM and ethidium bromide (Fig. 15.1b).

15.1.9

Organotypic Skin Equivalent Culture Model

The organotypic skin equivalent culture model is the most advanced and complex model currently available. The Fusenig lab was the first to use the organotypic skin model to study invasion of squamous cell carcinoma cells (Borchers et al. 1997). In simplest term,

an organotypic culture of skin can be engineered using its four major components, collagen, dermal fibroblasts, melanocytes, and epidermal keratinocytes (Parenteau et al. 1992). This model is ideally suited to study the effect of inhibitors or gene expression or UV on toxic effects or on invasive capability of tumor cells or melanocytes. These organotypic cultures have been extended to different tumor types including breast, prostate and ovarian cancer (Chioni and Grose 2008).

The experimental protocols for creating human skin reconstructs have been described elsewhere (Meier et al. 2000, 2003; Berking et al. 2001). In brief, for creation of an epidermal skin reconstruct, a mixture of collagen and human skin fibroblasts are seeded out into 6 or 24 well plates. After the fibroblasts have constricted the collagen, a mixture of keratinocytes and melanocytes are overlaid on top of the stromal layer. After the culture is established, the tray is lifted and the top exposed to the air, which induces keratinocyte differentiation. At maturity the human skin reconstruct has near identical histology to normal human skin and a basal membrane like structure is built (Meier et al. 2000, 2003) (Fig. 15.1c). For generation of a dermal skin reconstruct, one processes only until the fibroblasts have been seeded on the collagen layer (Sinnberg et al. 2010). When melanoma cells from the different defined tumor stages are introduced into the epidermal reconstruct, they exhibit progression-specific behavior. Cells derived from the radial growth phase melanoma, stay within the epidermal keratinocyte layer and do not breach the basement membrane (Meier et al. 2000, 2003). Cells from the vertical growth phase of melanoma invade through the basement membrane into the dermal fibroblast layer, and cells from metastatic lesions invade rapidly throughout the dermis (Fig. 15.1c). It is possible to include melanocytes or melanoma cells in which gene expression was modulated and it was shown that this can have dramatic effects on aggressiveness or invasive capability (Meier et al. 2003; Berking et al. 2001). The human reconstruct is a very useful model for modeling not only the growth of melanoma cells in a 3-D microenvironment, but the interaction of melanoma cells with the surrounding keratinocytes and fibroblasts. This approach can also be applied to other tissues as human breast, colon, and esophagus (Smalley et al. 2006b). Thus, the composite culture model enables functional studies of individual genes and interactions between specific gene products in various skin cell types in a biologically relevant milieu (Hsu et al. 2002; Meier et al. 2000; Sinnberg et al. 2010).

Skin reconstructs have a maximal life span of approximately 1 month *in vitro*, which can be extended to several months once grafted to living hosts (Javaherian et al. 1998; Satyamoorthy et al. 1999; Berking et al. 2001; Hsu et al. 2002). In these models, melanocytes regain their physiological localization at the level of the basal layer in the epidermis (Fig. 15.1c). They preserve all their functionality, since in response to UV rays they proliferate, synthesize, and secrete melanin (Auxenfans et al. 2009). Further advancements are the endothelialized skin equivalents in which human endothelial cells from umbilical vessels are seeded together with fibroblasts, which organize themselves into tubular structures with a well-defined lumen resulting in an endothelialized skin equivalent (Hudon et al. 2003; Auxenfans et al. 2009). Seeding of keratinocytes on this endothelialized dermis results in the formation of capillary structures (Velazquez et al. 2002; Smalley et al. 2006b). Finally, the immunocompetent skin equivalent model is available. The reconstruct is a novel 3-D culture system in which the migration of leukocytes toward tumor cells and the factors that influence leukocyte migration can be studied under *in vivo*-like conditions (Berencsi et al. 2007; Zhang et al. 2006).

15.2
Experimental Mouse Models

15.2.1
Introduction

The early development of metastases that are highly resistant to chemo- and radiotherapy is a fundamental characteristic of malignant melanoma. Experimental models in the laboratory mouse have been of critical importance to understand the biologic mechanisms how melanoma cells migrate in and out of blood and lymph vessels, evade immune defense, and colonize distant organs. In addition to the study of gene function on an organismal level, mouse models have also been widely used to evaluate the efficacy of novel treatment strategies in controlling metastatic disease progression. Experimental mouse models for cancer research can principally be subdivided into three different categories:

- Transplantation of human tumor cells into immunodeficient mice
- Transplantation of mouse tumor cells into syngeneic immunocompetent mice
- Primary autochthonous (“genetically engineered”) mouse models of cancer

We will briefly sketch the historical development of each category and provide a few selected examples how technical advances have enabled new insights in different areas of melanoma research over the years. We do not intend to provide a comprehensive catalog of all possible model systems but hope to give the reader a broad and stimulating overview (Table 15.2).

Table 15.2 Experimental mouse models

Melanoma model systems	Application	Advantages	Disadvantages
Transplantation of human melanoma cells into immunodeficient mice	Analysis of gene functions in human melanoma cells for invasive and metastatic tumor growth <i>in vivo</i> ; preclinical evaluation of new drug candidates	Fast, reliable assay that portrays important aspects of human melanoma biology	Excludes the role of immune cells in the tumor microenvironment, does not portray the early steps of malignant transformation
Transplantation of mouse melanoma cells into syngeneic immunocompetent mice	Analysis of gene functions in mouse melanoma cells for invasive and metastatic tumor growth <i>in vivo</i> ; preclinical evaluation of drug efficacy	Fast, reliable assay that includes the role of immune cells in the tumor microenvironment	Does not portray the early steps of malignant transformation, may not adequately portray all aspects of human melanoma biology

Table 15.2 (continued)

Melanoma model systems	Application	Advantages	Disadvantages
Primary autochthonous (genetically engineered) mouse melanoma models	Analysis of gene functions in the pathogenesis of melanoma on an organismal level; preclinical confirmation of drug efficacy	Allows for the investigation of early steps in malignant transformation in a fully immunocompetent tumor microenvironment	Time consuming and costly experimental setting, may not adequately portray all aspects of human melanoma biology

15.2.2

Transplantation of Human Melanoma Cells into Immunodeficient Mice

The successful engraftment of human tumors onto immunodeficient mice was first reported in 1969 using the athymic nude mouse (Rygaard and Povlsen 1969). This approach was widely used in subsequent years to study the morphological and biochemical characteristics of different tumor cells *in vivo* and the response of such xenografts to antineoplastic agents (Giovannella et al. 1973; Seaman et al. 1975). Several groups investigated the ability of human melanoma cells to grow metastatically in nude mice either following intravenous injection (“experimental pulmonary metastasis assay” or “lung colonization assay”) or following subcutaneous injection (“spontaneous metastasis assay”). In each case, the development of lung metastases was assessed over time. It was noted that melanomas, like most malignant neoplasms, were composed of heterogeneous tumor cell populations with different capacities for invasion and metastasis. Intrinsic features of a given tumor line appeared to be major determinants in regulating metastatic spread (Kozlowski et al. 1984). These features could be selected for by serial *in vivo* passaging. In addition, it was noted that growth of melanoma metastases was limited by residual immune reactivity of nude mice against xenogeneic human tumor cells, which was mediated particularly by NK cells.

Over the years, a number of melanoma cell lines were established (e.g., A375, MeWo, SKMel28, 1205Lu, and many others) which could be transplanted in nude mice as well as in mice with severe combined immunodeficiency (SCID), which were described a few years later. With the emerging ability to genetically modify tumor cells *in vitro*, these model systems have been successfully used to functionally assess the role of individual gene products on invasive growth and metastatic spread of melanoma cells *in vivo* until today. As an example, Clark et al. serially transplanted A375-M cells in nude mice and used cDNA microarray analyses to identify RhoC as a protein associated with melanoma metastases. Retroviral overexpression confirmed that RhoC indeed promotes the ability of melanoma cells to metastasize in mice *in vivo* (Clark et al. 2000). The ability to stably knock down the expression of a target gene using RNA interference has provided an additional valuable tool to study gene function not only on the cellular but also on the organismal level in mice. This technology was used in a recent paper to show that the CYLD protein regulates the ability of a human melanoma cell line to grow invasively and metastasize *in vivo* (Massoumi et al. 2009).

Several technical advances have expanded the range of experimental possibilities for the study of human tumor cells in immunodeficient mice. To more adequately model the tumor microenvironment, the group of Meenhard Herlyn transplanted human skin onto SCID mice and injected melanoma cells into the xenografts. This “orthotopic” tumor transplantation model circumvented the significant anatomic and physiologic differences between mouse and human skin and recapitulated the close interaction between melanocytes and keratinocytes (Juhasz Albelda et al. 1993). Crossing SCID mice onto the non-obese diabetic (NOD) genetic background improved tumor engraftment rates because NOD/SCID mice show reduced macrophage and NK function, as well as an absence of complement-dependent hemolytic activity. These mice were used by the group of Weinberg to study the *in vivo* growth properties of human melanocytes, which were transformed by defined genetic modifications *in vitro* (Gupta et al. 2005). Using this experimental approach, they provided evidence that the developmental origins of melanocytes in the neural crest might be relevant to their metastatic propensity. By combining both approaches it has today become possible to transplant complex skin reconstructs generated *in vitro* onto immunodeficient mice to study their behavior *in vivo* as mentioned in Sect. 15.1 above.

Another technical improvement for studying human tumor cells in immunodeficient mice has been developed by hematologists investigating the development and function of hematopoietic stem cells in immunodeficient mice. To further facilitate the permanent engraftment of bone marrow precursors, they tested various mice with additional genetic changes affecting the immune system. It was found that NOD/SCID mice, which lack the IL2 receptor common γ chain (NOD/SCID/ γ c mice), show extremely high engraftment rates using human hematopoietic cells Ito et al. (2002). The reason for the high engraftment rates were attributed to multiple immunological functional defects that affect dendritic cells in addition to the absence of T, B, and NK cells. Using these highly immunocompromised NOD/SCID/ γ c mice Carreno et al. demonstrated that the residual immunity in NOD as well as NOD/SCID mice affected the metastatic growth of A375 melanoma cells (Carreno et al. 2009). They used tumor cells stably expressing a luciferase gene and monitored tumor growth in the lungs in real time by measuring *in vivo* bioluminescence with a highly sensitive CCD camera. In addition, they demonstrated human melanoma recognition by residual NOD/SCID NK cells, which correlates with MICA/B expression and could be blocked by anti-mouse NKG2D antibodies.

The importance of using different immunodeficient mouse strains became prominently evident when analyzing the ability of different subpopulations of malignant cells derived from primary melanomas to generate tumor xenografts. The group of Markus Frank showed that only very few human melanoma cells can form tumors when transplanted into NOD/SCID mice, and that melanoma cells expressing the chemoresistance mediator ABCB5 were highly enriched for such “melanoma-initiating cells”. Importantly, they demonstrated that this minor tumorigenic cell population could be therapeutically targeted and destroyed *in vivo* with a specific monoclonal antibody (Schatton et al. 2008). Sean Morrison’s group showed that a slight modification of the xenotransplantation assay conditions and the use of more highly immunocompromised NOD/SCID/ γ c mice greatly increased the detection of tumorigenic melanoma cells (Quintana et al. 2008). The mechanisms underlying melanoma cell heterogeneity (which had already been observed in the

early days of melanoma xenografting) can now be studied using genetically modified transplantable melanoma cell lines in NOD/SCID/ γ c mice. The group of Meenhard Herlyn recently reported that a dynamically regulated JARID1B-expressing subpopulation of melanoma cells is essential for continuous tumor growth (Roesch et al. 2010). These results suggest a new understanding of melanoma heterogeneity with tumor maintenance as a dynamic process mediated by a temporarily distinct subpopulation. Highly sophisticated imaging techniques are currently being employed to monitor *in vivo* in real time the proliferation and migration of transplanted melanoma cells expressing various fluorescent and bioluminescent reporter genes placed under the control of constitutive or dynamically regulated promoters.

Taken together, the transplantation of human melanoma cells into immunodeficient mice can be generally considered as a rather rapid, reliable, and reproducible experimental approach to functionally study the role of genes in the process of malignant progression to metastatic disease. Importantly, this experimental system most closely reflects the intrinsic biology of human melanomas and partly recapitulates the tumor microenvironment *in vivo*. Furthermore, it is of critical importance for preclinical testing of novel therapeutics as was most recently demonstrated for small molecule inhibitors of mutated Braf (Yang et al. 2010a). However, work with human tumors in immunodeficient mice has two major inherent drawbacks: Firstly, it excludes the interaction of tumor cells with the immune system, which is of critical importance in shaping the tumor microenvironment and the process of tumor progression. Secondly, transplanted tumors grow rapidly and do not portray the early events during tumor initiation.

15.2.3

Transplantation of Mouse Melanoma Cells into Syngeneic Immunocompetent Mice

To experimentally include the role of the immune system in tumor progression requires the use of immunocompetent animals. However, in mice, melanoma develops only very rarely. One of these rare events occurred at the base of the ear in a C57BL/6 mouse at the Jackson Laboratories in 1954. This tumor was serially transplanted and subsequently established *in vitro* as the B16 melanoma cell line by Isaiah Fidler in the early 1970s. B16 melanoma cells readily form solid tumors when injected s.c. or i.v. into syngeneic C57BL/6 mice and were widely used from the early 1970s on as a model for metastases research to investigate the steps involved in tumor dissemination. Following the concept of “seed and soil”, originally proposed by Stephen Paget in the late nineteenth century (Paget 1889), Fidler and colleagues showed that both host factors and properties of the tumor cells contribute to the success or failure of the metastatic process. Already in 1977 it was reported that different B16 melanoma cell subclones derived *in vitro* varied greatly in their ability to form lung metastases upon intravenous inoculation into syngeneic mice. This observation suggested that B16 melanoma cells were heterogeneous and that highly metastatic tumor cell variants preexisted in the parental population (Fidler and Kripke 1977). The origins of tumor cell heterogeneity were also analyzed using K1735 melanoma cells, which were established from a UV-irradiated C3H mouse (Fidler and Hart 1982). These transplantable mouse melanoma cell lines are still used today to understand the cellular and molecular mecha-

nisms underlying the process of metastatic progression utilizing modern tools of molecular genetics to overexpress or silence individual genes and of *in vivo* imaging to follow fluorescent and bioluminescent reporter genes as described in the previous section.

The most important application of tumor transplantation models in immunocompetent mice, however, was to understand the role of innate and adaptive immune responses in tumor progression. Because B16 melanoma cells are poorly immunogenic and do not efficiently stimulate antigen-specific tumor immunity, they were widely used as a model to study tumor vaccine adjuvants, including various microbial extracts. Molecular insights into the mechanisms how immune responses against tumor cells develop was gained from studies with B16 melanoma cells genetically modified to secrete immunostimulatory cytokines. Unexpected at the time, expression of GM-CSF effectively promoted the induction of tumor immunity (Dranoff et al. 1993). It later became clear that this cytokine acted as a potent growth factor for antigen-presenting dendritic cells, which are critically required to initiate adaptive immunity. The experimental strategy to overexpress (or knock down) immune-related genes in B16 melanoma cells continues to reveal important insights into the interaction between tumor and immune cells until today. This is best illustrated by a recent report showing that the expression of the chemokine CCL21 by B16 melanoma cells induces a lymphoid-like reticular stromal network and recruits regulatory leukocyte populations to promote an immunotolerant microenvironment in mice (Shields et al. 2010).

The transplantable B16 melanoma model has also been used to study antigen-specific vaccination strategies including the use of synthetic peptides as well as recombinant proteins and genes. Initially, investigators targeted model foreign antigens such as chicken ovalbumin, which were stably transfected into B16 cells (Mayordomo et al. 1995). In our own work we found that efficient induction of cellular immune responses against clinically relevant melanocytic self-antigens such as TRP2 or gp100, which are naturally expressed by B16 melanoma cells, required strong activation of dendritic antigen-presenting cells either by culture from bone marrow precursors *in vitro* or by recombinant viral vaccines *in vivo* (Tüting et al. 1999; Steitz et al. 2000). Importantly, by combining antigen-specific vaccination with genetic modification of B16 melanoma cells we could show that the expression of type I IFNs promoted T cell effector functions in the tumor microenvironment (Steitz et al. 2001). Type I IFNs could also be efficiently stimulated in tumor tissue by adjuvant peritumoral injections of immunostimulatory nucleic acids such as polyI:C and CpG-rich DNA, which imitate viral RNA and DNA and activate the innate immune system through the Toll-like receptors (TLR) 3 and 9 (Tormo et al. 2006a).

The B16 model is also very suitable to study biological processes and therapeutic agents, which simultaneously affect both tumor and host cells, particularly immune cells. In addition to using genetically modified tumor cells, researchers have also employed genetically engineered hosts and injected specific monoclonal antibodies to dissect the underlying molecular and cellular mechanisms. Our own work with therapeutic oligonucleotides exemplifies this strategy. We explored the use of small immunostimulatory siRNA molecules, which simultaneously can silence a target gene in tumor cells, in our case the antiapoptotic gene Bcl2, and activate retinoic acid-inducible gene-I (RIG-I), a cytosolic sensor of viral RNA, in immune cells (Poeck et al. 2008). Silencing Bcl2 promoted tumor cell apoptosis and activation of RIG-I stimulated cytotoxic immune cells to

destroy tumor cells. Surprisingly, we found that activation of RIG-I also contributed to apoptosis induction directly in tumor cells. Experiments in mice, genetically lacking the interferon alpha receptor 1 chain, demonstrated that this response was critically dependent on a functional type I IFN system. Injection of cytotoxic antibodies against NK cells showed that this cell type largely mediated the antitumor response.

The interaction between melanoma cells and T cells in the tumor microenvironment *in vivo* can be studied in greater detail using adoptive lymphocyte transfer approaches. This experimental strategy has been facilitated by the development of T cell receptor transgenic mice which carry large numbers of genetically marked T cells that specifically recognize melanocytic antigens (Overwijk et al. 2003; Xie et al. 2010). The adoptive transfer and *in vivo* activation of T cells in mice bearing macroscopically detectable B16 melanomas enables investigations of the mechanisms that determine the balance between tumor regression and progressive disease, including the various possibilities of tumor immune escape. To more closely imitate the clinical situation, mice have been genetically engineered to express the human HLA-A2 molecule and T cells have been genetically engineered to express human T cell receptors (Frankel et al. 2010). These highly sophisticated experimental models have been of considerable help to translate this therapeutic approach into clinical reality for melanoma patients.

In summary, the transplantation of mouse melanoma cells into syngeneic immunocompetent mice also represents a rapid, reliable, and reproducible experimental approach to functionally study the role of genes in the process of malignant progression to metastatic disease. This experimental system may not fully reflect the biology of human melanomas but it much more adequately portrays the role of the immune system in shaping the tumor microenvironment *in vivo*. It has been of critical importance for preclinical development and testing of novel therapeutics, including melanoma vaccines with dendritic cells and immunomodulatory agents such as the anti-CTLA4 mAb, which is currently evaluated in clinical phase III studies. However, transplanted mouse melanomas also progress very rapidly following tumor inoculation and do not recapitulate the gradual series of cellular changes from premalignant to malignant pathologies.

15.2.4

Primary Autochthonous (Genetically Engineered) Mouse Melanoma Models

Advances in molecular genetics and stem cell biology have made it possible to study the functional role of oncogenes and tumor suppressor genes in the pathogenesis of melanoma on an organismal level. Work over the last decade has shown that genetic alterations observed in human melanomas also promote malignant transformation of melanocytes in mice. This most likely reflects the fact that the genetic control of melanocyte proliferation, migration, and differentiation in embryonic development is highly conserved during the evolution of vertebrates. Genetically engineered mouse tumor models initially involved the transgenic expression of viral or cellular oncogenes under the control of tissue-specific promoters. Transgenic expression of certain growth factors or growth factor receptors also promoted tumor development. Subsequently, it became possible to delete tumor suppressor genes by gene targeting. More recently, conditional deletion of tumor suppressor genes

or activation of oncogenes has been achieved via Cre-lox technology. Because genetically engineered mouse models for melanoma have been extensively reviewed (Chin et al. 2006; Larue and Beerman 2008; Zaidi et al. 2008), we will only briefly sketch their historical development and highlight some of the more important recent advances in the field.

Transformation of melanocytes in mice was first achieved by Beatrice Mintz's group who placed the oncogenic SV40 large T antigen under the control of a tyrosinase promoter construct specifically in melanocytes. Founder lines expressing high transgene levels developed eye melanomas very early while lines expressing low transgene levels developed eye melanomas much later. Grafting experiments of skin derived from mice with high melanoma susceptibility onto mice with low melanoma susceptibility revealed proliferating pigment cells close to areas of greatest wound healing, which subsequently evolved into invasively growing melanomas (Mintz and Silvers 1993). These observations strongly suggested that pro-inflammatory growth factors and cytokines known to be produced in wound repair can promote growth and malignant conversion of genetically susceptible melanocytes *in vivo*.

Lynda Chin and colleagues generated mice that expressed the activated Hras^{G12V} oncogene under a tyrosinase promoter in mice, which carried a deletion of the p16^{INK4a}/p19^{ARF} tumor suppressor gene locus (Chin et al. 1997). These mice spontaneously developed cutaneous melanomas after a short latency and with high penetrance. Her results indicated that activation of Ras and loss of p16^{INK4a}/p19^{ARF} can cooperate to accelerate the development of melanoma and provided the first *in vivo* experimental evidence for a role of p16^{INK4a}/p19^{ARF} deficiency in the pathogenesis of melanoma. In our own work, we could demonstrate the critical role of the p16^{INK4a}-Cdk4 interaction in suppressing the transformation of melanocytes following oncogene activation (Tormo et al. 2006b). Transgenic overexpression of the hepatocyte growth factor (Hgf) which drives Ras signaling via its receptor tyrosine kinase c-Met, cooperates with a mutated oncogenic cyclin-dependant kinase 4 (Cdk4^{R24C}) knocked into the germline, which abrogates p16 binding. Hgf-Cdk4^{R24C} mice spontaneously develop multiple small (benign) nevi and subsequently single progressively growing (malignant) melanomas, indicating that impairment of the p16^{INK4a}-Cdk4 axis affects the complete spectrum of stepwise malignant transformation of melanocytes (Landsberg et al. 2010).

With the recent development of techniques allowing for tamoxifen-inducible conditional activation of oncogenes specifically in melanocytes, it became possible to show that the mutated oncogenic Braf^{V600E}, which is frequently found in human melanomas, also strongly drives melanocyte proliferation in the skin of mice leading to the development of multiple melanocytic nevi (Dhomen et al. 2009; Dankort et al. 2009). Simultaneous deletion of the tumor suppressor genes PTEN or p16^{INK4a} led to a decreased latency and increased penetrance of melanoma. These novel models will undoubtedly help to dissect the molecular mechanisms regulating melanocyte proliferation, oncogene-induced senescence and tumor progression in the microenvironment of nevi and melanoma *in vivo*. The introduction of fluorescent or bioluminescent marker genes that are specifically expressed by melanocytes will enable the *in vivo* imaging of early proliferative events during melanocyte transformation, particularly following neonatal UV irradiation which has been shown to significantly promote melanomagenesis in several models, including Hgf-transgenic mice (Noonan et al. 2001).

Genetically engineered mouse melanoma models also provide novel opportunities to experimentally investigate the regulation of immune cell functions in the tumor microenvironment and understand the role of “cancer immunosurveillance” and “cancer-associated inflammation” in the pathogenesis of primary and metastatic melanoma. This is nicely illustrated by a recent report that primary melanomas developing in mice expressing the Ret oncogene as a transgene disseminate early but remain dormant for varying periods of time (Eyles et al. 2010). Control of tumor growth is mediated at least in part by cytotoxic T cells, since antibody-mediated depletion of these cells resulted in faster outgrowth of visceral metastases. These findings suggest that immune responses can be essential for prolonging the survival of early stage melanomas and that therapeutic strategies designed to reinforce such immune responses may produce marked survival benefits.

However, the immune system can be a double-edged sword because an inflammatory response can also promote tumor growth as has been first observed by Beatrice Mintz almost 20 years ago. Pro-inflammatory mediators capable of activating NF- κ B signaling may not only support tumor cell survival, regenerative proliferation, and migration but can also enhance neoangiogenesis. An important role for NF- κ B signaling in melanomagenesis has indeed recently been obtained by the group of Ann Richmond (Yang et al. 2010). They generated mice which allowed the inducible genetic ablation of I κ B β (a kinase which phosphorylates I κ B leading to NF- κ B nuclear translocation and activation), specifically in melanocytes expressing the oncogenic HRas^{G12V} mutation on the p16^{INK4a}/p19^{ARF}-deficient background. Deletion of I κ B β significantly inhibited the development of melanomas in these mice *in vivo* and promoted p53-dependent apoptosis and cell cycle arrest in cultured melanocytes *in vitro*. These results support a role for tumor cell-intrinsic activation of NF- κ B signaling in the pathogenesis of melanoma in agreement with reports in genetically engineered mouse models for other tumor types (Grivennikov et al. 2010). NF- κ B-driven inflammatory responses may also participate in the development of melanoma following burning doses of UV irradiation. This interesting question can now be experimentally addressed using UV-sensitive mouse models.

The impact of tumor-associated inflammatory responses on T cell-mediated immunosurveillance has been addressed by Anne-Marie Schmitt-Verhulst's group in mice where activation of oncogenic HRas^{G12V} and deletion of p16^{INK4a}/p19^{ARF} can be induced simultaneously by tamoxifen treatment (Soudja et al. 2010). These “TiRP” mice develop two different types of melanoma: slowly growing, pigmented melanomas and more rapidly growing, nonpigmented melanomas that are infiltrated by Gr1⁺/CD11b⁺ inflammatory immune cells. TiRP mouse melanomas also express the T cell-defined tumor antigen P1A in melanocytes, which allows investigations with adoptively transferred T cells specifically recognizing the P1A antigen on melanoma cells. Using this experimental system, it could be shown that an initially protective adaptive immune response is subsequently suppressed by chronic tumor-associated inflammation associated with infiltrating bone marrow-derived immature myeloid cells and a systemic Th2/Th17-oriented cytokine production profile.

In our own work we found that primary Hgf-Cdk4^{R24C} melanomas can effectively escape recognition and destruction by cytotoxic effector T cells. To experimentally introduce T cell-mediated immunosurveillance, we adoptively transferred melanoma-specific TCR-transgenic cytotoxic T lymphocytes in the context of a combination treatment protocol consisting of host preconditioning, viral vaccination, and adjuvant peritumoral injections

with immunostimulatory nucleic acids, which trigger innate immune responses in the tumor microenvironment (Kohlmeyer et al. 2009). This frequently resulted in complete and long-term regression of primary cutaneous melanomas. However, primary tumors eventually recurred indicating that some melanoma cells survived. Importantly, we observed large areas of poorly melanotic, proliferating melanoma cells in recurring melanomas suggesting that cytotoxic inflammation caused a phenotypic switch in subpopulations of tumor cells reminiscent of inflammatory melanomas in the TiRP mice (Landsberg et al. 2010). This phenomenon might represent a conversion of tumor cells toward a mesenchymal, invasive phenotype, which has been shown to result in the suppression of adaptive immune responses (Kudo-Saito et al. 2009).

Taken together, genetically engineered mouse melanoma models imitate the multistep pathogenesis of melanoma in man where primary tumor cells establish in a unique microenvironment, and naturally progress toward metastatic disease. These models are ideally suited to adequately address many of the key issues in melanoma biology including: the regulation of proliferation, differentiation, and senescence of melanocytes following oncogene activation; the origin of tumor cell heterogeneity and the mechanisms of phenotype switching; the relationship of melanoma cells with neural crest-derived precursor cells; the initiation of innate and adaptive immunity leading to immunosurveillance and inflammation; the role of pro-inflammatory mediators for tumor cell survival, proliferation and migration as well as neoangiogenesis; and the dynamics of tumor cell migration, dormancy, and metastatic colonization.

15.2.5

Experimental Mouse Models: Conclusions

A number of observations in the clinic suggest a close relationship between the activation of oncogenic signaling pathways driving malignant transformation of melanocytes and the activation of immune responses in the tumor microenvironment. Future work in different experimental mouse models will help to dissect the role of innate immune signaling pathways in tumor and immune cells which determine the balance between tumor regression and tumor progression. Even more importantly, work in these model systems will inform us how targeted inhibition of signaling pathways will affect the survival of tumor cells *in vivo* in the tumor microenvironment, including the response of immune cells. While genetically engineered mouse models adequately recapitulate many aspects of the early genetic events of melanocyte transformation, including the important role of the immune system in melanomagenesis, it must be kept in mind that they only partly reflect the features of human melanoma. Some of the more obvious shortcomings such as the low number of melanocytes in the interfollicular melanocytes may be overcome experimentally. Nevertheless, considerable differences will always remain in the molecular hardwiring of mouse tumor and immune cells that will have to be appreciated in each experimental setting. Furthermore, work with these model systems requires a long-term commitment since they are extremely time consuming, labor intensive, and costly. This may be alleviated in part by the generation of transplantable cell lines from primary melanomas with defined genetic alterations.

15.3

Final Remarks

In this chapter we have presented many of the commonly used complex *in vitro* cell culture model systems and the *in vivo* experimental mouse models that are employed by research groups today to study the various aspects of melanoma biology and to evaluate novel therapeutic strategies. The relative importance of each experimental approach depends largely on two important aspects: Firstly, how well does the model recapitulate the process it attempts to emulate? Secondly, how well does the model itself offer the flexibility to expand our knowledge relating to the pathologic process being evaluated? Today, researchers are more and more combining the strengths of new *in vitro* cell culture models, new “humanized” mouse strains for the transplantation of human melanomas, and new “genetically engineered” mouse models for primary melanoma to comprehensively analyze their particular aspect of melanoma biology.

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Abstract The emergence of selective BRAF inhibitors for the treatment of metastatic melanoma appears poised to change the therapeutic landscape in this disease. For decades the melanoma field has lagged behind others in achieving significant alteration in the natural history of metastatic disease with conventional cytotoxic chemotherapy. Even immunotherapy, a treatment standard both in the metastatic and adjuvant setting, fails to benefit more than a small minority of patients. While recent clinical trial results featuring novel immunologic therapies appear to be breaking the barriers observed with the previous generation of cytokine-based immunotherapies, the treatment of metastatic melanoma remains a severely unmet need. It is hoped that the discovery of somatic genetic alterations in signal transduction pathways that regulate proliferation, cell cycle, differentiation, and survival will provide further opportunities for expanding the reach of targeted therapy.

Keywords BRAF • MAP kinase pathway • cKIT • NRAS

16.1

Oncogenes Amenable to Direct Targeting

16.1.1

BRAF and the MAP Kinase Pathway

The identification of activating mutations in the serine threonine kinase, BRAF, in 50–60% of melanomas in 2002 was the watershed event that opens the door for investigation of molecularly targeted therapy in melanoma (Davies et al. 2002). BRAF is a constituent of the long studied MAP kinase pathway, a known mediator of growth factor signals in cancer (also refer to Chap. 7). However prior to the identification of BRAF mutations, only RAS mutations were known to constrictively activate the pathway in a subset of cancers, including melanoma. Therefore when BRAF mutations were screened for in a large series

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of diverse tumor types, and found, it was immediately apparent that these mutations might underlie the biologic importance of this pathway in 7–8% of all cancers. For reasons that remain poorly understood these mutations are found most commonly in melanoma and for that simple reason the laboratory investigation and clinical development of BRAF-targeted therapies are most advanced in this disease. The fundamental challenges that confronted the field were (1) understanding the potential vulnerability of BRAF inhibition despite the presence of numerous additional genetic alterations in the same tumors and (2) the development of potent and selective inhibitors.

In the years following the identification of BRAF mutations, sorafenib was the only targeted therapy in clinical development known to have some capacity for inhibiting RAF kinases including BRAF (Wilhelm et al. 2006). However, this agent had not been developed preclinically with the purpose of specifically targeting BRAF. Nonetheless, it provided the first opportunity to investigate the potential therapeutic relevance of BRAF mutations in melanoma and other cancers. Laboratory evidence suggested that sorafenib was able to have a cytotoxic effect on melanoma cell lines; however, this effect does not seem to depend on the presence or absence of BRAF mutations (Whittaker et al. 2010). This lack of cellular selectivity suggested the possibility that BRAF might be the important point of intervention even for tumors that lacked a RAF mutation. But, it was also known that nonpharmacologic methods for limiting the production of BRAF protein only impacted the growth and survival of melanoma cell lines with a BRAF mutation (Hingorani et al. 2003; Wellbrock et al. 2004). Clinical trials were rapidly designed and executed even before significant preclinical testing had occurred. Early in clinical testing it was revealed that sorafenib was not associated with tumor regression when administered as a single agent and only modestly inhibited the MAP kinase pathway in tumors analyzed during the first few weeks of therapy (Flaherty et al. 2005). This left open the possibility that more potent and selective BRAF inhibitors might yield superior molecular effects as well as evidence of clinical efficacy.

Several BRAF inhibitors have entered clinical trials within the past few years, each of which was developed and optimized in preclinical tumor models that harbor BRAF mutations. These agents fall into two classes: those that inhibit RAF kinases as well as others with near equal potency and those that are highly selective for RAF kinases. While the clinical trial results are either lacking or very immature for the less selective RAF inhibitors, two selective BRAF inhibitors have emerged from phase I clinical trials and demonstrate clear evidence of antitumor activity (Flaherty et al. 2010; Kefford et al. 2010). Even preclinically there are differences apparent between these two classes of RAF inhibitors. The less selective RAF inhibitors preferentially impact the growth and survival of cancer cell lines with BRAF mutations but show some evidence of efficacy in tumors with RAS mutations and even in some that lack either or RAF or RAS mutation (Venetsanakos et al. 2006; Mordant et al. 2010). The selective RAF inhibitors are distinguished from those agents by having very selective effects in BRAF mutant cancer cell lines (King et al. 2006; Bollag et al. 2010). This is evident at the level of MAP kinase pathway inhibition as well as growth inhibition in the induction of cell death. PLX4032 and GSK2118436 represent the first of these selective RAF inhibitors to have proven that BRAF is a valid therapeutic target (Flaherty et al. 2010; Kefford et al. 2010). This review details the results of the first-in-human clinical trials with these agents, which shared a focus on enrolling patients with BRAF mutant melanoma (Fig. 16.1).

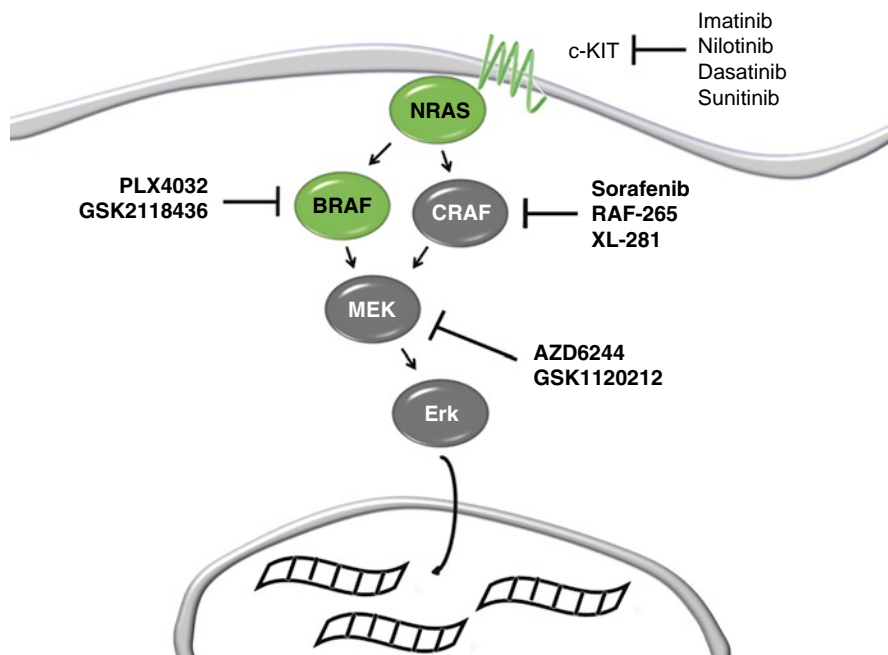


Fig. 16.1 c-KIT, RAF, and MEK inhibitors in clinical development for melanoma

In the phase I clinical trial of PLX4032, patients could have been accrued regardless of tumor type or BRAF mutation status (Flaherty et al. 2010). But, 49 of 55 patients enrolled during the dose escalation phase of the trial had metastatic melanoma and an increasing proportion of those were prospectively evaluated for the presence of a BRAF mutation as the trial progressed. Once the first evidence of objective response was manifest, the majority of patients subsequently accrued had BRAF mutant metastatic melanoma. This allowed not only for the characterization of safety and tolerability, but also an early exploration of efficacy. Even at doses that were not associated with significant toxicity the first responses were observed in the BRAF-mutant melanoma patients. As dose escalation proceeded to define the maximum tolerated dose, responses were even more frequently observed. Of the 21 metastatic melanoma patients enrolled to the highest five dose levels evaluated in the dose escalation phase of the trial, 16 of them harbored a BRAF mutation and 11 of them experienced an objective response by RECIST (the standard criteria for judging degree of tumor regression in solid tumor oncology trials). Additional patients demonstrated some evidence of tumor regression, but insufficient to be categorized as an objective response. In total, all but two of these initial 16 patients had at least minor evidence of tumor regression and remained on treatment following the first tumor response assessment. Dose-dependent toxicities that were commonly observed at the higher doses included rash, fatigue, arthralgia, and photosensitivity. Severe rash, fatigue, and arthralgia ultimately defined the maximum tolerated dose. The first resist responses were observed at 240 mg twice daily and the dose that exceeded the maximum tolerated dose was 1,120 mg twice daily.

After completion of the dose escalation portion of the phase I trial, 32 additional patients with BRAF mutant metastatic melanoma were enrolled at a dose level of 960 mg twice daily which was ultimately defined as the maximum tolerated dose (Flaherty et al. 2010). Twenty-six of these 32 patients achieved objective responses, two of which were complete. Four additional patients had minor degrees of tumor regression. The median progression-free survival for this cohort was estimated at greater than 7 months. Median overall survival had not yet been defined. At the recommended phase II dose rash, photosensitivity, and arthralgia continued to be commonly observed but were generally mild or moderate and could be easily managed with dose interruption or, when necessary, dose reduction. Nearly one-third of these patients experienced the development of one or more nonpigmented skin lesions during the initial months of therapy. When these lesions were biopsied and submitted for pathology, they were nearly always characterized as well-differentiated squamous cell carcinoma, squamous cell carcinoma/keratoacanthoma type, or simply keratoacanthoma. Patients were always able to continue treatment on protocol following removal of these lesions and most patients did not go on to have additional lesions. While it became clear that the emergence of these lesions was treatment-related, the mechanism underlying their emergence was not clear. The subsequent observation that selective BRAF inhibitors can stimulate MAP kinase pathway signaling in normal cells as well as cancer cells that harbor RAS mutations may belie the appearance of these lesions (Heidorn et al. 2010; Poulikakos et al. 2010). It has previously been described that a subset of keratoacanthomas and cutaneous squamous carcinomas harbor HRAS mutations (Corominas et al. 1989). Presuming then, that many melanoma patients have keratinocytes that have acquired HRAS mutations from prior sun exposure, the upregulation of the MAP kinase pathway induced by selective RAF inhibitors may facilitate their growth and clinical presentation.

GSK2118436 was evaluated in a phase I clinical trial of similar design to the PLX4032 study (Kefford et al. 2010). This agent is even more potent in inhibiting BRAF than PLX4032, able to inhibit mutant BRAF kinase activity by 50% at concentrations less than ten nanomolar. Ninety-three patients were accrued during the dose escalation portion of the trial, 85 of whom had metastatic melanoma, and of those 76 had activating mutations in BRAF. Of note, unlike the PLX4032 trial, in this study patients were eligible if their tumors harbored any mutation at the V600 position (including V600K and V600D). Doses ranged from 12 mg daily to 400 mg daily, in divided doses at the higher dose levels. At the highest dose, two dose limiting toxicities were observed: one case of severe pyrexia and one with syncope. However, even at this dose 14 of 15 patients were able to complete 1 month of therapy without interruption. Because there was no significant difference in drug exposure between 300 and 400 mg total daily dose, 150 mg twice daily was defined as the recommended phase II dose. Among 35 patients treated at one of these two highest dose levels, the most common toxicities were pyrexia, fatigue, rash, the appearance of nonpigmented skin lesions, headache, nausea, and vomiting. In nearly all cases these were mild to moderate in severity and did not require dose interruption. A subset of the skin lesions that manifested during treatment and were biopsied and found to contain well-differentiated squamous cell carcinoma, as was seen with PLX4032. Among the few patients treated at the two highest dose levels, there were ten objective responses observed (63% response rate). Only two patients demonstrated evidence of tumor size increase or progression at or before the first restaging evaluation. Of the six patients who were enrolled with V600K or

V600D mutations, three had objective responses. In addition to seeing responses in all visceral sites, several patients with small, asymptomatic, and previously untreated brain metastases were entered onto the study. In each case regression of these lesions was observed. The follow-up time for these patients treated in the last two cohorts of dose escalation was sufficiently short that median progression-free survival could not yet be estimated.

Taken together, the early clinical development of PLX4032 and GSK2118436 clearly confirm that BRAF is a valid therapeutic target when mutated in advanced melanoma. The early efficacy metrics of response rate and progression-free survival both appear clearly superior to available therapies for these patients. The completed, but not yet reported, phase II trial with PLX4032 seeks to corroborate the high response rate and robust median progression-free survival (NCT00949702). Single-agent chemotherapy, including dacarbazine or temozolomide, is associated with a 10–15% objective response rate and median progression free-survival of 2 months (Middleton et al. 2000). Combination chemotherapy regimens, including the recently studied carboplatin and paclitaxel, are associated with 15–20% response rates and median progression-free survival of approximately 4 months (Hauschild et al. 2009). High-dose interleukin-2 produces a 15% response rate and median progression-free survival of 2 months or less (Atkins et al. 1999). None of these therapies have been compared to best supportive care in an effort to demonstrate a survival advantage with these treatments. However, with median overall survival of the clinical trial populations ranging from 6 to 12 months for these therapies, it is not believed that they impact longevity were the population of patients treated as a whole. Clearly there are individual patients who can experience prolonged responses to therapy and do derive benefit. But, such patients make up such a small minority of all patients treated and investigational therapies have always been prioritized over administration of these standard treatments outside of the context of clinical trial. It is hoped, but not yet proven, that selective BRAF inhibitors will prolong survival compared to standard therapies. This is the subject of an ongoing phase III trial comparing PLX4032 to dacarbazine (NCT01006980).

Having documented clinical activity with selective BRAF inhibitors, the attention of the melanoma field has turned to understanding the molecular consequences of these therapies within melanoma and in normal tissues.

16.1.2

Combinations with Other Signal Transduction Inhibitors

In an effort to improve upon the results observed to date, a key question is what molecular and cellular processes mediate resistance to therapy. It has long been known that BRAF mutations are commonly accompanied by genetic alterations that activate the PI3K pathway and others that disrupt cell-cycle control at the level of p16/CDK4 (see Chaps. 4, 7, and 10). Given that these genetic alterations appear to be significant in melanoma pathophysiology, in conjunction with BRAF mutations, it is plausible that targeting components of these pathways while also inhibiting BRAF may overcome some of the intrinsic resistance to BRAF-targeted therapy. Selective inhibitors of PI3K, AKT, and CDK4 are themselves emerging from early-phase clinical trials.

In tumor biopsy samples collected from patients treated with PLX4032 and GSK 2118436, some degree of residual MAP kinase pathway activity persists even when patients are administered maximum tolerated doses. Similarly, residual ERK activity can be detected *in vitro* when BRAF-mutant cell lines are treated with high concentrations of the tool compound, PLX4720 (Paraiso et al. 2010). So, even before turning to combination regimens that target signal transduction pathways other than the MAP kinase pathway, there may be value in finding agents that inhibit the MAP kinase pathway at distinct points. MEK inhibitors may serve this purpose, and agents such as GSK1120212 have themselves shown single-agent clinical activity in patients with BRAF mutant melanoma. A phase I/II trial combining GSK2118436 and GSK 1120212 in patients with BRAF mutant melanoma is underway (NCT01072175). It is hoped that such a combination will suppress MAP kinase signaling to even greater degrees than are achievable with either agent alone. Additionally, it is hypothesized that the coadministration of a MEK inhibitor with a selective BRAF inhibitor will counter the upregulation of the MAP kinase pathway that occurs in normal tissues following selective BRAF inhibitor therapy. The consequence of dual BRAF and MEK blockade may be less toxicity, whereas the prediction is for most combination regimens to engender greater toxicity. While increased CRAF activity has been clearly documented in cells that lack a BRAF mutation (Heidorn et al. 2010; Poulikakos et al. 2010), the potential significance of this phenomenon in BRAF-mutant cancer cells has not been determined. It is possible that various resistance mechanisms could be facilitated by this mechanism.

It is not presently known if combination regimens that target multiple signal transduction pathways simultaneously will be tolerable to patients. And, there are additional strategies being considered for building upon BRAF inhibition in metastatic melanoma. These include combination regimens with anti-angiogenic therapy or immunotherapy. The combination of cytotoxic chemotherapy with anti-angiogenic therapy has been shown to be efficacious in a number of metastatic cancer populations (Hurwitz et al. 2004; Sandler et al. 2006; Miller et al. 2007). In the case of melanoma, BRAF-targeted therapy has far greater antitumor activity than conventional cytotoxic chemotherapy. Thus, combining BRAF inhibitors with anti-angiogenic agents may leverage some of the mechanistic interactions that have been described in preclinical systems such as normalization tumor vasculature and decreased interstitial pressure (Jain et al. 2007).

Combination of BRAF inhibitors with immunotherapies is an attractive approach for several reasons. First, BRAF does not appear to be an important constituent of the MAP kinase pathway in mature immune cells, including T cells (Tsukamoto et al. 2008). Second, recent evidence suggests that inhibition of mutant BRAF in melanoma cells results in upregulation of several surface antigens that have previously been described as being recognized by CD8-positive T cells (Boni et al. 2010). Third, antitumor immune responses can take several months to manifest in some patients treated with the new therapies such as ipilimumab, the time in which patients with aggressive metastatic disease can deteriorate clinically and succumb (Hodi et al. 2010). BRAF-targeted therapy may be able to forestall disease progression long enough for such patients to mount an antitumor immune response. Fourth, it has been demonstrated in preclinical models that destruction of tumor cells results in the release of higher amounts of tumor antigen which then facilitates one step in the process of effective immune activation (Correale et al. 2005).

Separate from the investigation of combination regimens including BRAF-targeted therapy, a better understanding of the mechanism of resistance that underlies delayed tumor progression following initial response will provide a basis for developing drugs and/or regimens that can be administered sequentially following single-agent BRAF inhibition. In preclinical systems, persistent, albeit reduced, MAP kinase pathway activity appears to underlie some portion of primary and secondary (or acquired) resistance (Paraiso et al. 2010). As noted above, an ongoing trial combining GSK2118436 and GSK1120212 (MEK inhibitor) is currently seeking to define a tolerable dose combination and will then investigate clinical activity. Such a combination could be hypothesized to increase the magnitude of initial response to therapy, perhaps including a higher complete response rate. If secondary resistance can be prevented or delayed by blocking the MAP kinase pathway at a point other than BRAF, then the same regimen may be associated with more prolonged responses.

16.1.3

PI3 Kinase Pathway

The PI3 kinase pathway is another RAS effector pathway that has been implicated in the pathophysiology of numerous cancers, including melanoma. Melanomas commonly feature loss of PTEN function, a negative regulator of AKT activation by PI3 kinase (Guldborg et al. 1997). Hemizygous and homozygous deletion appear to be the most common type of genetic alteration in PTEN in melanoma. Additionally, numerous point mutations, thought to inactivate PTEN, and thereby permitting higher PI3 kinase pathway activity, have also been described in some melanomas (Celebi et al. 2000). A point of continued controversy is whether silencing of PTEN expression by promoter hypermethylation might also be a mechanism of gene silencing in melanoma (Mirmohammadsadeh et al. 2006; Furuta et al. 2004). In any case, it is clear that a substantial subset of melanomas have genetic alterations in PTEN, and the preponderance of evidence suggests that it is the consequence of overactivity of the PI3 kinase pathway that is of pathophysiologic significance. It should be noted that numerous other cytoplasmic and nuclear sites of PTEN activity have been described, but it is unclear if any of those are important to melanoma biology (Zhang and Yu 2010). Some evidence has been produced from array comparative genome hybridization experiments to support the presence of AKT3 amplification in a distinct subset of melanomas from those that have PTEN loss, and rare activating mutations in AKT3 have been reported (Stahl et al. 2004; Davies et al. 2008). This is further genetic evidence that the PI3 kinase pathway plays a critical role in a subset of melanomas. Notably, it appears that PTEN loss is frequently seen concomitant with BRAF mutation (Tsao et al. 2004). This suggests that both of these RAS effector pathways are needed to contribute to melanoma formation.

Experimental evidence supports a role for the PI3 kinase pathway in melanoma invasion into the dermis, and potentially in metastatic spread. In particular, the introduction of activated AKT results in the conversion from a radial growth to vertical growth phase phenotype in primary melanoma in animal model (Govindarajan et al. 2007). Conversely, restoration of PTEN expression or blockade of PI3K/AKT signaling in melanoma cells

blocks invasion and induces apoptosis (Stewart et al. 2002; Ruth et al. 2006). Introduction of AKT3 into melanoma cell lines promotes proliferation and survival, whereas knock-down of AKT3 mRNA, but not AKT1 or AKT2, inhibits both phenotypes (Stahl et al. 2004). Introduction of myristylated AKT prevented expression of proapoptotic mediators such as Bim and Bmf and knockdown of AKT3 with siRNA greatly enhances the degree of apoptosis seen in BRAF-mutant cell lines treated with a selective BRAF inhibitor (Shao and Aplin 2010).

The development of therapeutic agents targeting the PI3 kinase pathway, and particularly the components within it that are critical to melanoma pathophysiology, is at a very early stage. Due to the overall prevalence of PTEN loss, activating mutations in PI3 kinase itself, and activating mutations or amplification in AKT, significant efforts have been made in the pharmaceutical industry to develop antagonists of this pathway. Three classes of PI3 kinase inhibitors have emerged in clinical development: (1) inhibitors of specific PI3 kinase isoforms, (2) agents that inhibit with near equal potency all PI3 kinase isoforms, and (3) dual inhibitors of PI3 kinase and mTOR. When considering which of these agents might be most relevant in melanoma, the deficiency in our knowledge regarding which isoform or isoforms of PI3 kinase to target becomes apparent. In lymphocytes, it appears that the catalytic subunit p110delta (the delta isoform) is the one whose activity is most markedly upregulated in the setting of PTEN deletion (Janas et al. 2008). However, in breast cancer models it appears that PI3 beta is the most relevant isoform in mediating cell invasion in response to RAS activation (De Laurentiis et al. 2011). These observations remain to be confirmed, specifically in melanoma.

The therapeutic strategy at the level of AKT may be somewhat more straightforward based on available genetic and experimental evidence. Given that the most common activating mutations in AKT across cancers are in AKT1, the focus of pharmaceutical development has been on agents with preferential inhibitory effects on this isoform (Carpten et al. 2007). AKT2 appears to be the most critical isoform in glucose homeostasis, and therefore would be the one isoform to avoid targeting (Altomare et al. 1998). The first generation of AKT inhibitors in clinical development has fairly uniform potency against the three AKT isoforms. This may be problematic, as dose-limiting toxicity may be rendered by AKT2 inhibition, or possibly coinhibition of all isoforms. The ideal AKT inhibitor for melanoma might be one that is relatively AKT3 specific, and such an agent has not yet been developed. In fact, the AKT inhibitor that is furthest into clinical development, MK-2206, is equally potent against AKT1 and AKT2, but fivefold less potent against AKT3 (Yan 2009). Thus, the melanoma field awaits the development of additional AKT inhibitors with more optimal properties relevant to the biology of AKT signaling in this disease.

16.1.4

CDK/Cyclin/p16

Germline mutation in CDKN2A, the gene that encodes both p16 and p14, have long been known to confer susceptibility to melanoma and is the highest penetrance allele yet discovered in familial melanoma (Hussussian et al. 1994). Mutations in CDKN2A, as well as hemizygous and homozygous deletions, are also observed in sporadic cases of melanoma (Wagner et al. 1998; Fujimoto et al. 1999). The best characterized point mutations identified

in familial melanoma result in a disruption of the p16/CDK4 inhibitory interaction, suggesting that this may be the critical interaction related to melanoma formation (Haferkamp et al. 2008). The net consequence of p16 loss or mutation is that CDK4 is dysinhibited and pushes cells through the cell cycle without the usual checkpoint regulation. In cases where CDKN2A is wild type, a distinct subset of melanomas harbor cyclin D amplification (Curtin et al. 2005). Cyclin D is another binding partner for CDK4 and is a critical coactivator of CDK4 activity. Thus, cyclin D application results in overactivity of CDK4, analogous to loss of p16 function. Finally, CDK4 itself has been found to be amplified in some cases of sporadic melanoma and to contain activating mutations in the kinase domain in another subset (Muthusamy et al. 2006). This genetic evidence would support that the CDK4 cell cycle checkpoint is a critical suppressor of melanoma formation and, conversely, its overactivity appears to contribute to melanoma pathophysiology. *In vitro* as well as mouse transgenic models support cooperation between activating BRAF mutations and genetic aberrations in p16/cyclin D/CDK4 (Dhomen et al. 2009; Robinson et al. 2010). What remains to be determined is whether dual targeting of this axis combined with an inhibitor of the MAP kinase pathway, such as BRAF, will have greater therapeutic value than inhibiting either target alone.

Restoring p16 function in the setting of mutation or deletion is beyond the scope of what current pharmacologic development can achieve. And, cyclin D lacks an enzymatic function against which drugs could be readily generated. That leaves CDK4, with its kinase activity, as a potentially relevant point of intervention in tumors with p16/cyclin D/CDK4 aberrations. Potent and selective CDK4 inhibitors have recently been developed and completed or are in the process of completing phase I clinical trials. An example of this class of drugs is PD032991, which is an orally available CDK4/6 inhibitor (Fry et al. 2004). As one might project for a cell cycle-targeted therapy, this agent produced mild to moderate suppression that was reversible during planned treatment interruptions in phase I (O'dwyer et al. 2007). This agent and other CDK4 inhibitors have not yet been tested clinically in patients with melanoma. It would be of particular interest to evaluate the efficacy of single-agent CDK4 inhibitor therapy in patients with p16/cyclin D/CDK4 alterations. Even if substantial single-agent activity is not observed in such a genetically preselected patient population, the genetic evidence supporting CDK4 dysregulation as being critical to melanoma formation would support further clinical investigation in combination with other approaches. Now that BRAF inhibitors have established their own evidence of efficacy, the most feasible approach would be to test a combination of a selective CDK4 inhibitor with a BRAF inhibitor in BRAF-mutant patients. Ideally, such a trial would at least retrospectively, if not prospectively, analyze p16, cyclin D, and CDK4 at the genetic level to determine if mutation, deletion, or amplification are preconditions for response to a CDK4-targeted therapy when given in combination.

16.1.5

KIT

By the time CKIT mutations were reported in melanoma in 2006, KIT had already been validated as a therapeutic target in gastrointestinal stromal tumor (GIST) (Demetri et al. 2002). And, subsequently, several second-generation KIT inhibitors established therapeutic

benefit in this population as well (Fig. 16.1) (Demetri et al. 2006). However, it was not known whether the somatic genetic changes that might accompany KIT mutation in melanoma, perhaps differently than GIST, might render melanoma more or less susceptible to single-agent KIT-targeted therapy. Presently, little is known about such genetic alterations in melanoma. And, the relative rarity of KIT mutations in the entire melanoma population has meant that there are relatively few tumor samples and melanoma cell lines harboring KIT mutations available for preclinical testing to address these issues.

Imatinib was the first KIT inhibitor to demonstrate single-agent activity in GIST, and was the first agent to be evaluated clinically in melanoma (Hodi et al. 2008). In the years prior to the discovery of KIT and mutations in melanoma, three phase II clinical trials were conducted with imatinib as a single agent in metastatic melanoma (Ugurel et al. 2005; Wyman et al. 2006; Kim et al. 2008). However, nearly all of the patients enrolled on trials did not have melanoma that arose from the clinical subtypes in which KIT mutations can be found, mucosal, acral/lentiginous, and those associated with chronic sun damage. So, although KIT mutation analysis was not performed in the context of these trials, one would estimate that very few, if any, of these patients had KIT mutations. Only one patient experienced an objective response in those trials and this patient had a prior history of a mucosal melanoma primary. In addition to activating mutations in KIT, amplification has also been described, and can occur in the presence or absence of mutation. So, the hypotheses addressed in clinical trials with KIT inhibitors in melanoma are whether mutation and/or amplification might serve as predictive markers for response to these therapies. It should be noted that many of the mutations in KIT described in melanoma overlap with those previously seen in GIST. Some of these mutations have been described as imatinib sensitive in the context of GIST, while others have not. And, novel mutations in KIT have been described in some cases of melanoma as well. Thus new questions remain to be answered in the context of KIT inhibitor trials in melanoma to understand not only whether mutations previously described as sensitive to existing drugs would be so in melanoma, but also to determine if amplification or novel mutations are similarly predictive of response to therapy.

A single patient with mucosal melanoma whose tumor was found to harbor an internal tandem duplication in the juxtamembrane domain of KIT was treated with imatinib and an objective response lasting for at least several months was observed (Hodi et al. 2008). This first report established that KIT-targeted therapy could be effective in melanoma, but many questions remained.

Two phase II trials with imatinib in KIT mutant or amplified melanoma have been initiated and preliminary results reported. In one of those trials, Carvajal and colleagues screened 146 patients with metastatic melanoma whose primary tumors were mucosal, acral/lentiginous, or arose from chronic sun-damaged skin (Carvajal et al. 2009). Archival tumor specimens were screened for the presence of amplification in KIT or mutations in exons 9, 11, 13, 17, and 18. Twenty of 146 (14%) of these patients were found to have mutations in KIT, and an additional 4 (3%) had amplification only. A subset of those patients were found to satisfy the other eligibility criteria for the phase II trial and treatment was initiated with the highest dose used in GIST (400 mg twice daily). Fifteen patients (6 acral, 7 mucosal, and 2 CSD) were treated, of whom 12 were treated long enough to undergo a response evaluation. Two patients experienced complete responses and two had partial responses (33% response rate), all of which are ongoing with

follow-up ranging from 3 to 9 months. An additional 6 patients had disease stabilization at the first response evaluation; however, 4 of those patients had subsequent disease progression within 6–12 weeks. Three of the four objective responses were seen in patients whose tumors harbored an exon 11 L576P mutation, previously described as a magnet sensitive in GIST and the other had an exon 13 K642E mutation. Two patients with amplification only experienced short-lived stable disease. All of the responding patients were still on therapy at the time of this report, so median duration of response could not be defined.

In another phase II trial Guo and colleagues enrolled 35 patients with KIT mutation in exons 9, 11, 13, 17, and 18 or amplification only (Guo et al. 2010). The starting dose of imatinib is 400 mg daily, with escalation of the dose to 600–800 mg daily upon disease progression. Thirty-five patients were found to harbor these genetic alterations and were enrolled (16 acral, 10 CSD, 2 superficial spreading/nodular, 2 mucosal), 30 of whom had a response evaluation by the time of the initial report. Six patients (20%) had an objective response. In analyzing response by the KIT exon in which mutations were found, 2 of 12 patients with exon 11 mutations responded along with 3 patients with exon 13 mutations. Notably, 8 of the 12 patients with exon 11 mutations had disease stabilization (in addition to the 2 partial responders). Nine patients had disease progression, escalated the dose of the MAP to 600 or 800 mg daily, but only one achieved short-lived disease stabilization thereafter. One of three patients with amplification only had an objective response, and none had disease stabilization. Six patients underwent tumor biopsy at the time of disease progression, three of whom had had objective responses and three who had disease stabilization as their best response. In each case, the same KIT mutation was found at baseline and at progression. In one case, a BRAF^{V600E} mutation and a nonsense mutation in PDGFRalpha were found at the time of disease progression. Follow-up time was too short in this trial to estimate median duration of response or progression-free survival.

Based on these two trials several preliminary conclusions can be made: (1) the previously described imatinib-sensitive mutations in GIST also predict responsiveness to imatinib in melanoma, (2) several patients with predicted imatinib sensitivity did not respond, and (3) on the basis of a single response among the few patients treated with amplification only, KIT-amplified tumors remain a subgroup worthy of further investigation.

Additional phase II trials are underway with dasatinib and sunitinib in KIT inhibitor-naïve patients and nilotinib in patients who have progressed or who are intolerant of first-line KIT inhibitor therapy. Preliminary results from these studies are not yet available. The only randomized study to be initiated in this area, aiming to demonstrate not only objective responses but also overall disease control is a phase III trial comparing nilotinib to dacarbazine in treatment-naïve metastatic melanoma patients whose tumors harbor mutations in exon 9, 11 or 13, or Y822D, D820Y, or Y823D mutations in exon 17. Progression-free survival is the primary endpoint. The proof of concept has been established that KIT-mutant melanoma can be responsive to imatinib. However, much work remains to be done to understand the response rate for particular mutations, or in particular exons. KIT amplification alone remains of uncertain significance with regard to connoting responsiveness to therapy. And the durability of response or disease stabilization and its impact on overall survival remains to be determined. The pace with which these questions can be answered is limited by the rarity of genetic alterations in KIT in the overall melanoma population, and their presence in a minority of patients with the relevant clinical subtypes in which they can be found.

16.2

Oncogenes Not Readily Amenable to Direct Targeting

16.2.1

NRAS

Activating mutations in NRAS continue to be an elusive target in melanoma, more than 25 years after their initial identification (Albino et al. 1984). Approximately 20% of all advanced melanoma cases harbor an activating mutation in NRAS, with Q61R mutation being the most common and mutations at position 12 in the amino acid sequence being the next most commonly affected (Curtin et al. 2006). NRAS is not amenable to targeting with drugs of the sort that can compete with ATP binding as RAS does not consume ATP as an energy source. Rather, RAS is a GTPase, and the common mutation found in cancer impairs the GTPase activity. Thus, an effective pharmacologic inhibitor of mutated RAS would need to restore the lost GTPase activity, and such compounds have not been readily discovered to date. Experimental evidence demonstrating the biologic significance of RAS in melanoma stems from siRNA knockdown experiments as well as genetically engineered models demonstrating its contribution to melanoma pathophysiology (Eskandarpour et al. 2009; Nogueira et al. 2010). More indirect evidence supporting the therapeutic potential of antagonizing RAS comes from experiments using farnesyltransferase inhibitors, which block one of the key post-translational modifications required for RAS to localize to the plasma membrane (Smalley and Eisen 2003). The introduction of mutated NRAS into melanocytes induces senescence or transformation in the appropriate genetic background (Whitwam et al. 2007). In transgenic models, introduction of activating NRAS mutations combined with either p53 or p16 loss of function through genetic inactivation results in proliferative and invasive melanocytic lesions (Nogueira et al. 2010). In cell lines, knock-down of NRAS mRNA results in growth arrest and apoptosis selectively in melanoma cell lines that harbor activating NRAS mutations (Eskandarpour et al. 2009). At exposures that inhibit the farnesylation of many signaling molecules, farnesyltransferase inhibitors will arrest the cell cycle and induce cell death in melanoma cells that harbor NRAS mutations. And, this intervention sensitizes such cells to the cytotoxic effects of chemotherapy. Unfortunately, chemical trials with farnesyltransferase inhibitors have been largely disappointing, owing to the lack of evidence of single-agent efficacy in patients with RAS-mutated cancers when these agents are administered at maximum tolerated doses (Gajewski et al. 2006). While there is some evidence that partial inhibition of RAS signaling can be achieved with these agents, it appears that the degree of innovation is insufficient to significantly perturb these tumors (Sebti and Hamilton 2000). Thus, agents with more selectivity for RAS are anxiously awaited, but for the reasons noted above continue to be a major technical challenge.

An alternative strategy would be to block the downstream signal transduction pathways that are activated in the setting of RAS mutation. There are numerous RAS effector pathways, the MAP kinase and PI3K pathways being the best described, but the relative importance of each has not been elucidated. There is recent evidence that RalGDS may be an important pathway downstream of RAS as well (Mishra et al. 2010). As drug targeting of

RAS effector pathways has been a major priority in pharmaceutical development, there are numerous agents emerging targeting the MAP kinase and PI3K pathways which might be useful for NRAS-mutant melanoma. In particular, potent and selective MEK inhibitors have demonstrated single-agent activity in BRAF mutant melanoma and might serve as relevant agents to be used in combination in NRAS mutant melanoma (Infante et al. 2010). In the PI3K pathway, it is still unclear what the optimal point of intervention is, with PI3K itself, AKT, and mTOR all being constituents of the pathway for which small molecule inhibitors have been developed. Currently there are combinations of MEK and PI3K or AKT inhibitors being evaluated for safety in phase I trials. The primary focus of these development efforts is KRAS-mutant cancers, which are far more common in the overall cancer population than NRAS-mutant tumors. However, as these combination regimens emerge from phase I testing they will likely be evaluated in NRAS-mutant melanoma as an initial approach.

16.2.2

MITF

The microphthalmia transcription factor, MITF, is the master regulator of melanocyte differentiation and expression of melanocyte-specific antigens (see also Chap. 2 Abdel-Malek). Its potential role as an oncogene in melanoma has recently been suggested based on the discovery of high-level amplification of the MITF locus in approximately 15% of melanomas (Garraway et al. 2005). In such tumors, and some others without amplification, knockdown of MITF with siRNA impairs proliferation. Being a member of the transcription factor superfamily that includes MYC, a contribution to cancer pathophysiology would not be unprecedented. However, targeting MITF with pharmacologic inhibitors is not currently possible. This molecule lacks an enzymatic domain against which small molecules could readily be developed. Rather, agents that block the association of MITF with cofactors, or key promoter regions in MITF target genes, would be needed. In order to avoid pleiotropic effects on the expression of a vast array of genes, likely resulting in unacceptable toxicity, a deeper understanding of which MITF target genes contribute most significantly to melanoma biology is required. Such a detailed understanding would potentially generate alternative targets that are amenable to pharmacologic therapy. In the meantime, there is evidence that epigenetic regulation of MITF may be apparent in the setting of cancer and could be ameliorated to some degree with the use of the histone deacetylase inhibitor (Yokoyama et al. 2008). A clinical trial is currently ongoing in metastatic melanoma patients using a class of specific histone deacetylase inhibitor (LBH589).

16.2.3

GNAQ/GNA11

In 2008 activating mutations in the G protein-coupled receptor signaling molecule, GNAQ, were first described in a large subset of uveal melanomas (Van Raamsdonk et al. 2009). The same mutations are not found in other melanoma subtypes. The following year,

analogous mutations in the highly homologous GNA11 were described in an additional portion of uveal melanoma cases. In total either of these mutations appears to be present in approximately 80% of uveal melanomas. It had previously been shown that BRAF and NRAS mutations are not found in uveal melanoma, so the discovery of these activating mutations filled significant void in the understanding of oncogenic drivers in this clinical subtype of melanoma. Like RAS, these proteins are GTPases, in which the GTPase activity is disabled by the mutation that had been described. Therefore, pharmacologic agents would need to restore the GTPase activity in order to antagonize the signaling capacity of these molecules. As has been noted, such an approach appears to be extremely technically challenging, and such candidate therapies do not currently exist.

Outside of the context of cancer, these G protein alpha subunits (GNAQ and GNA11) are known to activate several downstream signal transduction pathways, including the MAP kinase and PI3 kinase pathways (Van Raamsdonk et al. 2004). Early experimental evidence in melanoma cell lines harboring a GNAQ mutation suggests that the MAP kinase pathway may be a point of vulnerability for therapeutic purposes (Van Raamsdonk et al. 2009). Given the availability of potent and selective MEK inhibitors for clinical evaluation in this setting, clinical valuation of this hypothesis is quite feasible. Two such clinical trials are underway currently employing single-agent MEK inhibitor therapy, but results are not yet available. Further preclinical work is needed to understand more about the hierarchy of signal transduction pathways downstream of these G proteins so that rational combination drug strategies can be formulated.

16.2.4

BAP1

The most recent genetic discovery in melanoma also comes in the uveal melanoma sub-population. It has long been known that the short arm of chromosome 3 is frequently deleted in uveal melanomas, and particularly those that metastasize and result in fatality (van Gils et al. 2008). However, a potential candidate tumor suppressor gene on this chromosome had not been described. Recently, massively parallel exome sequencing has uncovered mutations in BRCA1-associated protein 1 (BAP1) in more than 80% of metastatic uveal melanomas (Harbour et al. 2010). The distribution of these mutations in regions of the gene/protein that interacts with two distinct deubiquitinases, UCH and ULD, suggest that BAP1 is indeed a tumor suppressor gene. As with any other tumor suppressor gene in cancer, we currently lack the ability to restore the function that is lost through inactivating mutations or deletion. However, a greater mechanistic understanding of the activities of BAP1-binding partners may provide opportunities for therapeutic intervention. The two deubiquitinases that the genetic evidence suggests to be important in BAP1 function are likely not ideal therapeutic targets as ubiquitination and deubiquitination are fundamental levels of regulating protein stability that pharmacologic inhibitors of such molecules may have far-reaching and toxic effects. One observation made in previous mechanistic studies regarding BAP1 function suggests that the BAP1 regulates the activity of the polycomb repressive complex 1 (PRC1) which regulates histone acetylation and the acetylation (Scheuermann et al. 2010). Experimental evidence suggests that in

Table 16.1 Oncogenes and tumor suppressor genes in melanoma and therapeutic strategies

Oncogene	Pathway	Drug targeting strategy
C-KIT	RAS	Direct, ATP competitive kinase inhibitors
NRAS	RAS	Combinations targeting RAS effector pathways
BRAF	MAP kinase	Direct, ATP competitive kinase inhibitors Downstream, allosteric MEK inhibitors
AKT3	PI3 kinase	Direct, allosteric AKT inhibitors
CDK4	p16/RB	Direct, ATP competitive CDK4 inhibitors
Cyclin D	P16/Rb	Indirect, ATP competitive CDK4 inhibitors
MITF	Melanocyte differentiation	Indirect, histone deacetylase inhibitors
GNAQ/GNA11	G-protein-coupled receptors	Indirect, PKC, MAP kinase, PI3 kinase pathway inhibitors
Tumor suppressor gene	Pathway	Drug-targeting strategy
PTEN	PI3 kinase	PI3, AKT, mTOR inhibitor
p16	p16/RB	Indirect, ATP competitive CDK4 inhibitors
BAP1	polycomb repressive complex 1	Indirect, histone deacetylase inhibitors

BAP1-mutated uveal melanoma cell lines, histone deacetylase inhibitors have a growth inhibitory effect (Harbour et al. 2010). This approach is certainly possible to investigate in clinical trials, as numerous histone deacetylase inhibitors have been developed.

16.3 Conclusions

The discovery of somatic genetic changes in oncogenes and tumor suppressor genes, and subsequent biological validation of their significance has opened the door for the development of molecularly targeted therapies aiming to antagonize the very genetic underpinnings of melanoma (Table 16.1). The proof of principle has been established with BRAF inhibitor treatment that melanoma can be vulnerable to such an approach, despite the vast array of genetic alterations present within anyone tumor. However, it is equally clear that no single point of intervention will eradicate metastatic melanoma, and that understanding the hierarchy of oncogenes and signal transduction pathways becomes critical to develop rational combination therapies which might make an even more significant impact. Numerous challenges and hurdles remain, including the fact that restoring the functions

that are lost through inactivation of tumor suppressor genes requires indirect therapeutic strategies seeking to antagonize the pathways that are activated as a consequence. With each point of potential therapeutic intervention, the concern regarding the normal physiologic function of these targets and pathways must be considered. For single-agent treatment strategies, such as BRAF, some degree of effect on signaling within normal tissues is manageable. However, combination targeted therapy strategies will certainly require that each component within the combination be highly selective for its intended target, or that the target is uniquely relevant to melanoma biology and not normal physiology. It remains possible that individual molecularly targeted therapies will exert their best effect when given in combination with therapeutics of other classes, including anti-angiogenic or immunologic. Given our increasingly detailed understanding of the network of genetic changes and aberrant signal transduction and the downstream phenotypic consequences on various cellular processes, there is no shortage of essential points of intervention or types of combinations. It will be critical for the melanoma field to remain mindful of the ultimate goal, which is to tailor the therapeutic approach to the individual patient based on the unique constellation of alterations within their tumor.

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Abstract After decades of phase III trials failing to demonstrate an impact on survival by cytokine-based regimens and the lack of progress in the development of therapeutic vaccines in patients with metastatic melanoma, novel ways of modulating the immune system by monoclonal antibodies have changed the landscape. For the first time a clinically important and statistically significant impact on survival by a systemic therapy in advanced metastatic melanoma patients has been developed. Immunotherapy has finally established itself and is here to stay. The impact of ipilimumab-based immunotherapy in patients with advanced melanoma is a new corner stone for many subsequent developments. Other novel monoclonal antibodies are being developed and substantial hope is emerging that these developments may be able to provide a future for the so far stagnant development of effective therapeutic vaccines in metastatic disease.

In the adjuvant setting, vaccines have performed remarkably poorly. In a number of trials multiple vaccinations seemed even associated with worse outcome. A similar boost to vaccine development, also in the adjuvant setting, may depend on combinations with innovative immunomodulators such as ipilimumab. A new development is also the relative IFN-sensitive profile discovered in patients with ulcerated primary melanomas and low tumor burden in large adjuvant EORTC trials. Here, we may finally target therapies for selected patient populations.

Keywords Melanoma • Immunotherapy • Ipilimumab • Interferon • Cellular therapy • Vaccin

17.1

The End of the Cytokine-Chemotherapy Era

Advanced metastatic melanoma is almost invariably incurable, with a median survival time of only 6–9 months and a 3-year survival rate of only 10–15% (Balch et al. 2009). In spite of increased response rates with 4–5 drug biochemotherapeutic regimens, survival

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benefits were not demonstrated compared to DTIC monotherapy (Eggermont and Kirkwood 2004). This sobering experience is exemplified by the outcome of no less than 21 phase III trials evaluating the addition of cytokines as IFN- α or of IL-2 or their combination to mono or combination chemotherapy (Eggermont and Schadendorf 2009). In Europe, DTIC is the only approved drug. Fotomustine is approved in only some countries on the basis of a phase III trial delaying the onset of brain metastases (Avril et al. 2004). In Europe, IFN and IL-2 are not approved for advanced metastatic disease, whilst In the USA, IL-2 is approved based on a compilation of phase II studies in 277 patients, showing lasting complete responses in 7% of patients (Atkins et al. 1999). Cytokine-based immunotherapy was unable to deliver and a long era of rather fruitless and toxic biochemotherapeutic regimens ended in the first decade of the twenty-first century.

17.2

Innovative Immunomodulation

17.2.1

Anti-CTLA-4 Antibodies: Inhibit the Inhibition

An immune signal will only be generated when an antigen is presented by an MHC molecule and a co-stimulatory molecule, B7.1 or B7.2, binds to CD28 to provide the signal for T-cell activation. Downregulation of this process is simultaneously initiated by B7-CTLA4 binding. Thus, monoclonal antibodies that bind to CTLA-4 can block the interaction between B7 and CTLA-4 resulting in the inhibition of this negative switch, which may break peripheral tolerance to self-tissues and induce antitumor responses (Inman et al. 2007). Two fully human IgG monoclonal antibodies recognizing CTLA-4, ipilimumab (MDX-010), and tremelimumab (CP-675,206) have been tested, alone or in combination, in phase II/III trials.

17.2.2

Clinical Activity of Ipilimumab

Already early in its development program it became clear that Ipilimumab can induce long-lasting responses (Weber et al. 2008). A 13% response rate with Ipilimumab in combination with a vaccine was reported in a randomized phase II study in 56 patients (Attia et al. 2005) and better clinical responses were observed in patients with grade 3/4 autoimmune toxicity, an observation confirmed in a study of 139 patients at the Surgery Branch of the NCI (Downey et al. 2007). The most common grade 3/4 immune-related adverse events were colitis/diarrhea and dermatitis that responded to systemic steroids without significantly affecting the efficacy of ipilimumab therapy (Beck et al. 2006). A more aggressive regimen than the dose of 3 mg, the early studies was then evaluated with an induction phase consisting of 10 mg/kg every 3 weeks for 4 months (Q3Wx4) along with a maintenance treatment of 10 mg/kg ipilimumab every 12 weeks starting at week 24.

This intensive regimen has emerged as the most effective schedule in a large randomized phase II trial comparing doses ranging from 0.3 to 3 mg to 10 mg/kg (Wolchok et al. 2010). It has been used in most further phase II and phase III clinical trials including the pivotal trial of DTIC versus DTIC+ipilimumab in first line in stage IV patients as well as in the EORTC18071 adjuvant trial in stage III. This schedule showed clinical activity, in the form of either objective response or stable disease, in 27% of 155 patients with metastatic melanoma who developed progressive disease on a median number of 2 prior therapies. Median OS was 10.2 months and immune-related grade 3/4 adverse events occurred at 21.9% of patients. In 2009, a remarkable 2-year overall survival was reported of >50% for patients receiving ipilimumab in first line and of about 30% in patients receiving ipilimumab in second line (Schadendorf et al. 2009). Overall positive results of ipilimumab in patients with metastatic melanoma led to the initiation of a pivotal trial in first line comparing dacarbazine±ipilimumab. Another phase III trial of ipilimumab alone or in combination with a gp100-peptide vaccine in second line was reported at ASCO in 2010 and simultaneously published in the NEJM (Hodi et al. 2010). A remarkable survival benefit for the ipilimumab containing treatment arms was observed. Survival at 2 and 3 years were almost doubled in the ipilimumab containing treatment arms in comparison to the vaccine-alone treatment arm. It thus seems likely that ipilimumab will be the first drug in the history of melanoma that will be approved on the basis of a demonstrated survival benefit in patients with advanced metastatic melanoma. It also means that the pivotal trial in first line comparing DTIC versus DTIC+Ipilimumab will also be most likely successful and dramatically change therapeutic options for metastatic melanoma patients. Interestingly, ipilimumab-mediated benefits are not limited to restricted patient populations on the basis of mutations, HLA-types, LDH serum levels, or other tumor-related factors (Schadendorf et al. 2009). Thus we are witnessing a very significant new era in melanoma where immunotherapy has established itself and is here to stay.

17.2.3

Tremelimumab

In contrast, the development of the second anti-CTLA4 monoclonal antibody in the field of melanoma has ended, at least for now, after an initially equally promising start. Early phase clinical studies of tremelimumab demonstrated acceptable similar efficacy of 10 mg/kg monthly and 15 mg/kg quarterly doses (Camacho et al. 2009). A 8.3% response rate in a large phase II study database of 246 patients single agent tremelimumab (15 mg/kg quarterly) was reported with an interesting quality of the responses of 6+ to 18+ months and a median OS of 10.1 months (Kirkwood et al. 2010). Yet, when this schedule was evaluated in comparison with standard dacarbazine or temozolomide chemotherapy in first line, the trial was stopped early for futility (HR 1.04) (Ribas et al. 2008). At longer follow-up and further analysis, it was shown that patients with a CRP < 1.5 upper normal limit seemed to derive a significant survival benefit from treatment with tremilimumab. A phase III trial in this limited patient population was planned, but later canceled when it had become obvious that ipilimumab was active in all subgroups of patients (Hodi et al. 2010) and is expected to establish itself also for all patients in first line.

17.2.4

Improved Response Criteria on Endpoints for Immunotherapy

Important is the observation that new lesions in patients receiving ipilimumab or tremelimumab may not always indicate progressive disease and treatment failure as defined by WHO criteria. Four patterns of response have been observed: (a) response in baseline lesions; (b) stable disease with slow, steady decline in total tumor burden; (c) response after initial increase in total tumor burden; (d) response in index and new lesions after the appearance of new lesions. Importantly, in 7 out of 26 patients who developed new lesions after 12 weeks of treatment, regression or stabilization of disease was observed (Wolchok et al. 2009). Novel, immune-related response criteria (irRC) may more accurately describe response to immunotherapy and avoid premature treatment cessation in patients with disease progression prior to response. Contrary to mWHO criteria, irRC (a) only considers measurable lesions (>1 cm), (b) defines total tumor burden as the sum of index lesions identified at baseline and new lesions detected after baseline, and (c) aims for follow-up after progressive disease to detect late activity (Hoos et al. 2010).

17.2.5

Anti PD-1

Another monoclonal antibody that has been developed acts against the programmed death-1 receptor (PD-1R), the ligand of which (PD-1L) can be directly expressed on melanoma cells. PD-1R is a part of the B7:CD28 family of costimulatory molecules that regulate T-cell activation and tolerance and thus anti-PD-1R can play a role in breaking tolerance (Butte et al. 2007; Wong et al. 2007). Recently, the first clinical results of this approach in a multiple tumor phase I trial, in which a number of melanoma patients were included, were published and indicated activity that seems similar to anti-CTLA4 antibodies (Brahmer et al. 2010).

17.2.6

Agonistic Antibodies OX 44 and Anti-CD137 (4-1BB)

The antibodies anti-OX44 and anti-4-1BB have an agonistic action on T-cell activation and the anti-CD25 antibody that targets T-regulatory cells that constitutionally overexpress CD25 (Melero et al. 2007). Combinations of these antibodies can significantly optimize T-cell responses which indicate that a whole new field of immunomodulation is emerging (Gray et al. 2008). Phase I dose-escalation study of BMS-663513, an agonist anti-CD137 human monoclonal antibody, in 54 metastatic melanoma patients reported manageable toxicity with fatigue, transaminitis, and neutropenia being the most common adverse events, and clinical activity that justifies its further development both as a single agent and in combination (Sznol et al. 2008).

17.2.7

New Vaccine Developments in Advanced Metastatic Melanoma

The development of an effective therapeutic vaccine for metastatic melanoma continues to be the elusive “holy grail” in a disease where now signaling-pathway inhibitors and anti-CTLA4 antibodies are emerging as effective therapies. The good news is that the anti-CTLA4 and other immunomodulatory antibodies may come to the rescue of the vaccine development field, as they may play a crucial role in maintaining an immune response initiated by a vaccine.

A number of advances however should be discussed here.

A recombinant MAGE-A3 fusion protein combined with different immunological adjuvants – AS02B or AS15 – has been assessed in the EORTC 16032–18031 randomized phase II trial as a first-line treatment to 68 patients with unresectable stage III or stage IV M1a melanoma. The combination with AS15 yielded higher anti-MAGE-3 antibody titers, stronger T cell induction and some long-lasting clinical responses (Kruit et al. 2008). A gene signature derived from pretreatment tumor biopsies has been developed and shown to predict clinical benefit (Louahed et al. 2008). A randomized trial in patients with resected stage IIIB and IIIC melanoma (DERMA) is ongoing and serves at the same time as a validation trial for the prognostic, hopefully predictive, gene profile.

A randomized phase III trial comparing vaccination with the gp100:209–217(210M) peptide in combination with high dose IL-2 with treatment with IL-2 alone was reported to improve PFS significantly without a clear impact on survival (Schwartzentruber et al. 2009).

Considerable interest has been generated by the 28% overall response rate observed in a recent phase II study with intratumoral injections of OncoVEX^{GM-CSF} – an oncolytic herpes simplex virus vector encoding granulocyte monocyte colony-stimulating factor (GM-CSF) – into 43 stage IIIC and IV patients (Senzer et al. 2009). Injected tumors routinely responded often with local complete response, within 2 months of therapy. Importantly, systemic long-term responses were observed independent of the disease stage: 6 CR, 6 PR, 7 SD of injected tumors. The induction of systemic immunity has been demonstrated (Kaufman et al. 2010). A pivotal phase III trial in 360 previously treated, unresectable melanoma patients has now reached full accrual and will be evaluated end of the year 2011 or beginning of 2012 (Kaufman and Bines 2010). Another pivotal vaccination trial with intratumoral gene delivery is ongoing in M1a patients with Allovectin-7®. This is a plasmid/lipid complex containing the DNA sequences encoding HLA-B7 and β 2 microglobulin, which together form a major histocompatibility complex, or MHC, class I. In general, vaccine development strategies may benefit most from combinations with novel immunomodulating monoclonal antibodies.

17.2.8

An Update on Adoptive Immunotherapy Developments

The adoptive immunotherapy program of Rosenberg and coworkers at the Surgery Branch of the NCI has provided new insights and better results over the years. The most important recent developments have demonstrated that conditioning the patient by mild myeloablative

chemotherapy with cyclophosphamide and fludarabine will induce lymphopenia under which conditions the adoptive transfer of T-cell clones derived from the tumors of the patient will expand vividly *in vivo* after transfer into the patient and lead to overall response rates of 50% with over 10% CRs (Dudley et al. 2008). These results have recently been confirmed by the experience in Tel Hashomer in Israel (Besser et al. 2010). In the NCI program, it was further demonstrated that when total body irradiation is added to precondition the patient further, to eliminate or reduce also the lymphocyte populations in the bone marrow that would compete for IL-2, response rates as high as 72% have been observed with 16% CRs (Dudley et al. 2008). Confirmatory studies outside the NCI are ongoing.

Conversion of normal peripheral blood lymphocytes (PBL) into antitumor lymphocytes by transduction with genes encoding one of the T-cell receptor (TCR) relevant to tumor cells is another development in adoptive immunotherapy. Rosenbergs' group reported on 4 tumor responses in 31 patients with metastatic melanoma treated by autologous PBL transduced by MART-1-specific TCR following mild myeloablative conditioning (Morgan et al. 2006).

17.2.9

Immunotherapy in Melanoma in the Adjuvant Setting

With the modest exception of interferon, the history on the development of adjuvant immunotherapy regimens in the adjuvant setting of melanoma is remarkably sobering and in conflict with the hypothesis that immunotherapy should be easier to develop and be established in the adjuvant setting with minimal tumor load than in the advanced immunosuppressed metastatic patient.

Some 25 randomized trials, almost invariably underpowered, have been conducted in stage II/III melanoma to evaluate adjuvant therapies, such as chemotherapy, nonspecific immune stimulants such as BCG (bacillus Calmette-Guerin), *Corynebacterium parvum*, levamisole, or combinations of these agents with dacarbazine chemotherapy (Eggermont and Gore 2007).

17.2.10

Interferon Alpha (IFN)

IFNalpha-2b and IFNalpha-2a have been explored in the adjuvant setting in stage II and stage III melanoma patients in multiple phase III trials over the last 25 years, which indicates that results are rather multi-interpretable (Eggermont 2001). The various trials in which treatment with IFN was compared to observation are summarized in Table 17.1. Adjuvant therapy regimens with IFN can be divided in high dose IFN (HDI), intermediate dose IFN (IDI), and low dose IFN (LDI). HDI trials typically use doses of 10-20MIU/M2, IDI trials of 5-10 MIU as a flat dose, and LDI trials 3MIU as a flat dose, with a single trial going as low as only 1 MIU.

The table shows that overall hazard ratios (HR) are rather comparable between the three dose levels. Also it is clear that the impact on disease-free survival (DFS) is rather

Table 17.1 IFN versus observation in stage II/III patients

Trial	Stage	Treatment	DFS	OS
<i>High dose IFN</i>				
NCTCG (Creagan et al. 1995)	II-III	IFN α 2a, 3 \times 20MIU/M2/wk, im, 3 mts	5-yr; HR=0.77; p =0.19	5-yr; HR=0.88; p =0.40
ECOG 1684 (Kirkwood et al. 1996)	IIB, III	IFN α 2b, 20 MIU/M2qd1-5/iv, + 3 \times 10MIU/M2/wk, sc, 48 wk	6.9 yr; HR=0.56; p =0.0046	6.9 yr; HR=0.68; p =0.046
ECOG 1690 (Kirkwood et al. 2000)	IIB, III	IFN α 2b, 20 MIU/M2qd1-5/iv, + 3 \times 10MIU/M2wk, sc, 48 wk	4.4 yr; HR=0.90; p =0.054	4.4 yr; HR=1.07; p =0.99
SUNBELT (McMasters et al. 2008)	III SN+	IFN α 2b, 20 MIU/M2qd1-5/iv, + 3 \times 10MIU/M2/wk, sc, 48 wk	5.3 yr; HR=0.82; p =0.46	5.3 yr; HR=1.03; p =0.90
<i>Intermediate dose IFN</i>				
EORTC 18952 (Eggermont et al. 2005)	IIB-III	IFN α 2b, 10 MIU/qd1-5/sc, 4 wk +3 \times 10MIU/wk, sc, 12 mts or +3 \times 5MIU/wk, sc, 24 mts	4.65-yr; HR=0.81; p =0.12	4.65-yr; HR=0.88; p =0.40
Nordic (Hansson et al. 2007)	IIB, III	IFN α 2b, 10MIU/qd1-5, sc, 4 wk +3 \times 10MIU/wk, sc, 12 mts or 3 \times 10MIU/wk, sc, 24 mts	6 yr; HR=0.83 ; p =0.05	6 yr; HR=0.88; p =0.47
<i>Low dose IFN</i>				
French (Grob et al. 1998)	II	IFN α 2a, 3 \times 3MIU/wk for 18 mts	5-yr; HR=0.75; p =0.035	5-yr; HR=0.72; p =0.059
Austrian (Pehamberger et al. 1998)	II	IFN α 2a, 3MIU/qd, 3 wk +3 \times 3MIU/wk, 12 mts	3.4 yr; HR=0.62; p =0.02	3.4 yr; HR=0.83; p =NS
Scottish (Cameron et al. 2001)	IIB, III	IFN α 2b, 3 \times 3MIU/wk, 6 mts	2-yr; HR=0.72; p =0.05	2-year; HR=0.81; p >0.2

(continued)

Table 17.1 (continued)

Trial	Stage	Treatment	DFS	OS
ECOG 1690 (Kirkwood et al. 2000)	IIB, III	IFN α 2b, 3 \times 3MIU/wk, for 24 mts	5 yr; HR=0.90; $p=0.17$	5 year; HR=0.93; $p=0.81$
UKCCR (Hancock et al. 2004)	IIB, III	IFN α 2a, 3 \times 3MIU/wk, for 24 mts	5 yr; HR=0.94; $p=0.6$	5 yr; HR=0.91; $p=0.3$
WHO-16 (Cascinelli et al. 2001)	III	IFN α 2a, 3 \times 3MIU/wk, for 36 mts	5 yr; HR=0.95; $p=0.5$	5 yr; HR=0.96; $p>0.5$
German (Garbe et al. 2008)	III	IFN α 2a, 3 \times 3MIU/wk, for 24 mts	4-yr; HR=0.69; $p=0.018$	4-yr; HR=0.62; $p=0.0045$
EORTC 18871 (Kleeberg et al. 2004)	II-III	IFN α 2b, 3 \times 1 MIU/wk, for 12 mts	8 yr; HR=0.96; $p>0.5$	8 yr; HR=0.96; $p>0.7$

im intramuscularly, *iv* intravenously, *sc* subcutaneously, *MIU* million international units, *qd* every day, *mts* months, *wk* week, *yr* year, *HR* hazard ratio

consistent and always clearly better than the impact on overall survival (OS). Meta-analyses have demonstrated this and indicate a consistent effect on DFS but a very small effect on OS, of at best 2.8%, without a clear differential effect on outcome by dose or length of treatment (Wheatley et al. 2003, 2007). A similar outcome was obtained in the EORTC 18991 trial, comparing long-term therapy with pegylated IFN to observation in stage III patients (Eggermont et al. 2008). In this trial, the impact on relapse-free survival (RFS) was significant in the ITT population. The impact in the sentinel-node positive patients was most pronounced and significant not only for RFS, but also for distant metastasis-free survival (DMFS). Ulceration of the primary seems to indicate a different biologic entity that may be more sensitive to adjuvant therapy with IFN (Wheatley et al. 2007; Eggermont et al. 2009). Moreover, the two largest trials, EORTC 18952 and EORTC 18991 in 2,644 patients clearly demonstrated that also patients with stage IIB or with only microscopic positive (only sentinel node-positive) stage III disease seemed to benefit from adjuvant IFN or pegylated IFN, whereas in more advanced stage III patients the benefit was absent or negligible (Eggermont et al. 2009). For the EORTC Melanoma Group the observation that IFN effects were by far the best in patients with ulcerated primaries lead to the decision to perform an adjuvant trial with pegylated IFN compared to observation in node-negative patients with ulcerated primaries, the EORTC 18081 trial.

The lack of benefit observed in stage IIIB/C with adjuvant IFN therapy was for the EORTC Melanoma Group. This was the reason to move to a different drug. Thus, the EORTC 18071 trial, comparing a 3-year treatment with ipilimumab to placebo in a double blind randomized setting, was activated in 2009 and is expected to complete accrual in 2011.

17.2.11

Vaccines in the Adjuvant Setting

Remarkably, in the setting believed to provide the best opportunity for therapeutic vaccine development, results have been most disappointing thus far. A number of trials even indicate that vaccines can be harmful.

Of six large, randomized trials of allogeneic melanoma cell-based vaccines conducted, three trials failed to demonstrate a benefit (Hersey et al. 2002; Sondak et al. 2002; Wallack et al. 2010). Two large trials with Canvaxin, an allogeneic tumor cell-based vaccine, resulted in an early stopping of the trials because of a negative trend for the vaccine arm in resected stage IV patients and a significant detrimental outcome for the vaccine in stage III patients (Morton et al. 2007). Two large trials with the GMK vaccine (ganglioside GM2/KLH/QS-21) resulted in an early stopping of the trials because of inferior outcome in the vaccine arms compared with high dose IFN (HDI) in the ECOG1694 trial (Kirkwood et al. 2001) in stage IIB/III patients and compared with observation in the EORTC 18961 trial in stage II patients (Eggermont et al. 2008). So, the Canvaxin trials and the EORTC 18961 trials clearly evoked fears that prolonged, multiple administration of vaccines could potentially be harmful (Eggermont 2009a, b). These fears have been somewhat appeased by the final evaluation of the EORTC 18961 trial (Eggermont et al. 2010), which no longer shows this detrimental effect on survival in the ITT analysis. However, in the per protocol analysis a detrimental outcome for the vaccine arm is still significant. Thus, with these clear worries the final results of the canvaxin trials are eagerly awaited.

Regarding the adjuvant use of GM-CSF, a recent report of the ECOG4697 trial at the ASCO 2010 annual meeting failed to demonstrate a significant impact on survival (Lawson et al. 2010). There are some reports that GM-CSF as part of a vaccine can potentially make outcome worse. In two randomized trials, the GM-CSF containing arms did worse than the vaccine alone arms (Faries et al. 2009; Slingsluff et al. 2009). These observations indicate that multiple vaccinations can potentially be harmful but thus far unidentified, potentially immunosuppressive, or tolerance-inducing mechanisms. It has been speculated that the novel ways to block the activity of T-regulatory cells by anti-CLTA4 antibodies may be an important strategy to overcome the woes of therapeutic vaccine development in melanoma (Eggermont et al. 2008b, 2009a).

17.3

Conclusions

A new era in melanoma immunotherapy development has arrived. Ipilimumab is the first drug to demonstrate a clear and clinically highly significant survival benefit in melanoma patients with advanced metastatic disease. Therefore, the ongoing pivotal adjuvant trial in stage IIIB/c patients, with ipilimumab, the EORTC 18701 trial, comparing ipilimumab to placebo in a double blind randomized setting is fully justified, and its results will be awaited with great anticipation.

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